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FURTHER IMPROVEMENTS IN THE PARTIAL-VACUUM METHOD OF INOCULATION WITH LOOSE SMUT FUNGI [1]

W. J. Cherewick and R. H. Cunningham [2]

SUMMARY

The effectiveness of inoculation of wheat and barley with loose smut fungi by Moore's partial-vacuum method can be increased by selection of spikes in the proper stage of development, repetition of the evacuation process 3–5 times, and use of a power-operated pump. The optimum stage of development for inoculation is at the time the earliest flowering florets contain ovaries approximately ½ the size of mature kernels. Most of the florets are inoculated when evacuation and sudden release of air pressure is repeated from 3 to 5 times.

A power-driven portable pump was developed to overcome the pressure variation and strenuous work that accompanies hand pumping. The unit consists of a vacuum pump and a 1/20 H.P. electric motor geared down to operate the pump at 54 strokes per minute.

In the course of studies on physiologic specialization in the fungi causing loose smut of barley and wheat, *Ustilago nuda* (Jens.) Rostr. and *U. tritici* (Pers.) Rostr. (1, 2), it became apparent that Moore's partial-vacuum method of inoculation (5) was suitable for handling a large number of smut collections. With improvements in the method (3) that permitted inoculation of 10 or more heads at a time, it became possible to inoculate, on the average, over 1700 plots each season by means of 3 pump outfits. Moore (5) and other investigators (4, 8, 9) found that the partial-vacuum method of inoculation was less effective on barley than on wheat. With the addition of the sponge rubber insert in the split-stopper to stop leakage (3), it was possible to get over 90 per cent infection in some susceptible varieties of barley. The variability in the amount of infection obtained in barley during different periods or seasons of inoculation, however, was nearly as high as that reported by Middleton and Chapman (4), ranging from 39 to 91 per cent in a susceptible variety.

TIME OF INOCULATION IN RELATION TO FLOWERING.—Pickenbrock (7) observed that the most favorable period for wheat inoculation was that from shortly after pollination until 5–7 days later. Shands and Schaller (8), using their needle method, inoculated 2 varieties of barley, Wisconsin Barbless and Oderbrucker, at different stages of development and obtained good infections from inoculations from 1 day before to 3 days after pollination. Inoculation by the partial-vacuum method gave best results on the day of pollination.

The exact time of pollination in barley is difficult to determine, since it occurs within closed florets and frequently while the spikes are enclosed in the boot. The opening of the florets and extrusion of the anthers at anthesis is readily observable, however, and this stage of development was used at Winnipeg as a measuring point in the inoculation tests.

Two susceptible varieties of barley, 6-rowed Plush and 2-rowed Sanalta, were inoculated at various stages of flower development, ranging from about 3 days before anthesis to the time when the kernels were from ½ to ¾ the mature size. The length of this period varies from 5 to about 15 days depending on variety of barley and on weather conditions. Inoculations were made by 2 methods: 1) injecting a spore suspension into each floret with a hypodermic needle and 2) Moore's improved partial-vacuum method.

With the partial-vacuum method, it was impossible to force spore suspension into very young florets while the lemma and palea were still soft and flattened (Fig. 1, B). The earliest effective inoculation by this method could be made 1 or 2 days before anthesis when the glumes began to harden and round off (Fig. 1, A). The results of a representative inoculation test are summarized in Table 1.

Other varieties of barley and some varieties of wheat have been tested and gave similar results. These results indicate that barley, as well as wheat, can be inoculated with loose smut fungi from a few days before anthesis to the time the grains develop ½ their full size. Inoculation of very young florets, however, injures a high proportion of the ovaries so that little seed is set. Seed that develops after such inoculation produces weak seedlings, many of which die during early stages of growth. Inoculation during a period of from about a day before anthesis to the time when the grains reach ½ their mature size does not normally interfere with seed development, and the inoculated seed germinates as well as the noninoculated. The effectiveness of inoculation during this period depends on the proportion of florets in the inoculated spikes that receives the spore suspension; however, even in 6-rowed barley varieties that have a considerable spread in time of flowering within a single spike (9, 10), the length of the flowering period does not exceed that of susceptibility. In other

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Contribution No. 1513 from the Botany and Plant Pathology Division, Science Service, and Contribution No. 201 from the Cereal Crops Division, Experimental Farms Service, Canada Department of Agriculture, Ottawa, Ontario.

[2] Plant Pathologist, Plant Pathology Laboratory, Winnipeg, Manitoba, and Technician, Cereal Breeding Laboratory, Winnipeg, Manitoba.

FIG. 1. Two spikes of barley; A) About a day before anthesis showing glumes in central florets well rounded. B) More than 3 days before anthesis with glumes soft and flattened.

words, spikes in which the earliest florets contain grains ¼ to ½ the size of mature kernels usually have their youngest florets in anthesis or in a stage shortly before anthesis and, therefore, are in the optimal stage for inoculation by partial-vacuum method.

METHOD OF INTRODUCING SPORE SUSPENSION INTO FLORETS.—The basic principle of Moore's original partial-vacuum method for inoculation with loose smut fungi (5) consisted of alternate evacuation of the inoculation chamber and release of air pressure with each stroke of the pump. Moore recommended 4–6 strokes for wheat and 10–15 or more for barley. Oort (6) modified the method so that each consecutive stroke of the pump exhausted more air from the chamber. He used a single evacuation with 10 strokes of the pump and obtained over 80 per cent infection in a susceptible variety of barley. The modified method was used for several years at Winnipeg but later was further modified (3) to increase the capacity of the chamber and to reduce air leakage by the insertion of sponge rubber into the split stopper. From 6 to 10

TABLE 1.—*Percentage of smut[a] in 2 varieties of barley following inoculation by 2 methods at different stages of development*

Stage at inoculation	Hypodermic needle method		Partial-vacuum method	
	Plush	Sanalta	Plush	Sanalta
Glumes soft, flat, and coherent	78	82		
Glumes tough and rounded off	81	84	69	84
Anthesis stage	84	81	73	83
2 days after anthesis	87	89	76	88
Grains approximately ½ normal size	73	85	81	85

[a] Percentage of smutted heads in 6-ft.-long plots.

strokes of the pump created a vacuum of 25–30 in. of mercury.

In the inoculation tests described in the foregoing paragraphs, examination of the inoculated spikes revealed that only florets at anthesis contained the spore suspension after a single evacuation process. Florets with tightly closed lemma and palea remained dry. Repeating the operation 3 or 4 times eventually forced spore suspension into all florets except the very young ones, which had soft and flattened glumes.

Pumping faster to increase the vacuum or pumping longer to increase the period of vacuum had no effect on the proportion of florets that received spore suspension. It seems, therefore, that besides evacuating as much air as possible from the florets it is necessary to loosen the tight coherence of the palea and lemma to permit the entrance of the spore suspension. The alternate evacuation and sudden release of air pressure apparently is effective in freeing the tight clasp of the flowering glumes. With some varieties of barley it is helpful to roll the spikes lightly between the palms of the hands or to manipulate the spikes with the fingers before inoculation.

In the method of inoculation used at Winnipeg for common wheat varieties and the more easily inoculated barley varieties there are 3 alternate evacuations and sudden releases of air. On varieties of barley and some durum wheat that are more difficult to inoculate, air is evacuated and released 5 times.

THE MECHANICAL PUMP.—Moore's partial-vacuum method (5, 6) of inoculation required several strokes of the hand-operated pump for wheat and 10–15 strokes for barley. In modifications at Winnipeg, the inoculation chamber was increased in size to 5 cm inside diameter to accommodate 10 heads of wheat or barley at a time. In this apparatus, up to 10 pump strokes were necessary to create sufficient vacuum. With 3–5 alternate evacuations and releases of air pressure, it required 30–50 pump strokes for each setting. The operation of the hand pump was, therefore, a very strenuous task. Moreover, different pump operators could not work with identical force, nor could they continue with the same force during different hours of the day. Any variation in the

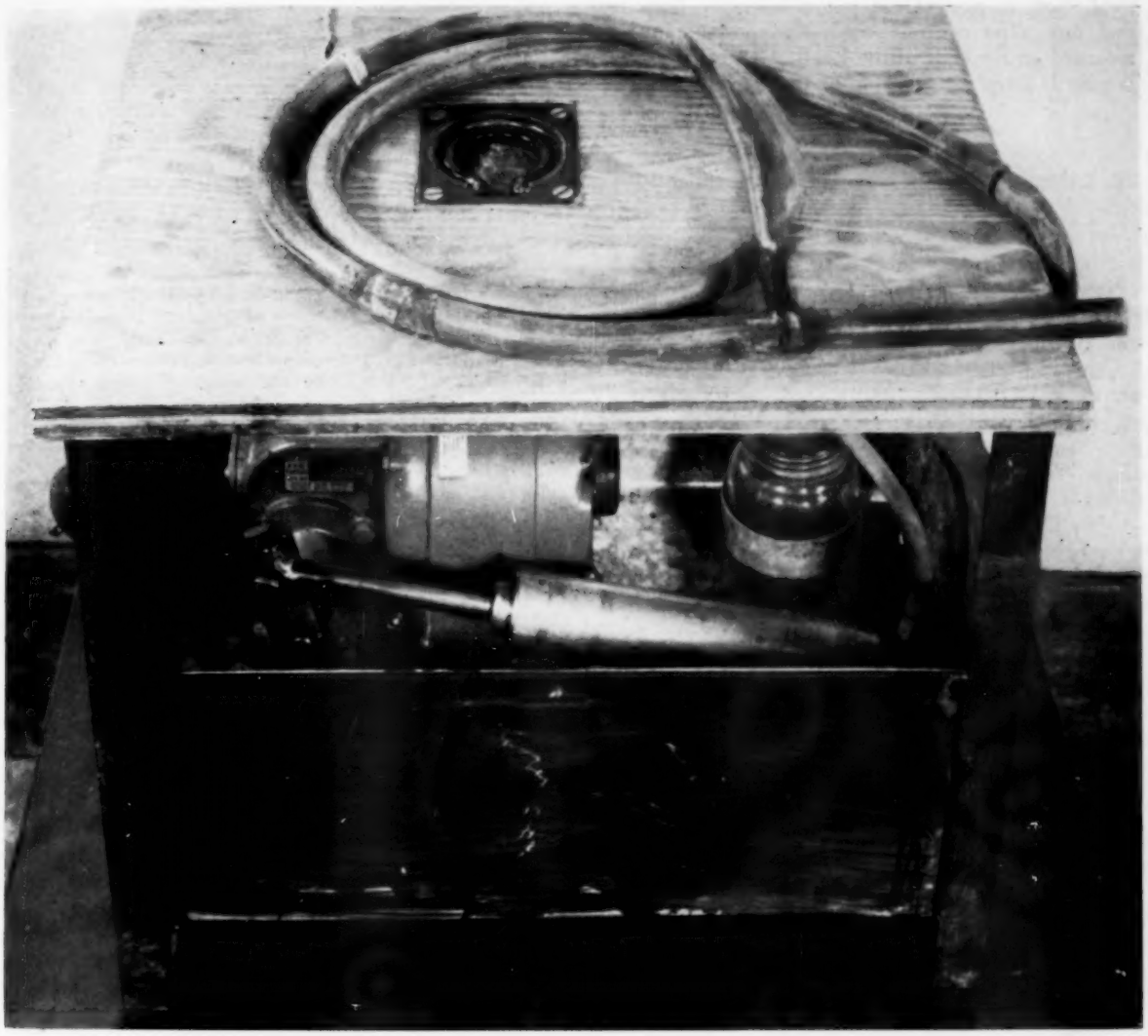

FIG. 2. A stool pump for loose smut inoculations by power-operated pump.

speed and force of pumping caused wide differences in the amount of infection obtained, even in the same variety of barley. To overcome such difficulties it was necessary to develop a power-operated pump.

DESCRIPTION OF THE PUMP.—The stool pump described here (Fig. 2) is intended for use with a portable gasoline-powered alternating-current generator. It consists of a Boston gearhead motor of 1/20 H.P. with a horizontal output shaft that rotates at 54 rpm. A crank arm with a throw of 2.5 in. is attached to the output shaft of the motor. This gives the pump a 5-in. stroke. The crank arm carries a crank pin to which is attached the plunger shaft of the vacuum pump. The pump was made from a piece of brass tubing, 1.5 in. in diameter and 7 in. long, with internal threads cut in each end. A brass guide-block for the plunger rod is screwed into the top end of the cylinder; a brass block, containing a check valve and connection for the vacuum hose, is screwed into the bottom end. The plunger is made from 2 brass washers and a leather cup washer in the same manner as an ordinary tire pump plunger except that the cup washer is turned for suction on the upstroke of the pump. The lower brass block is drilled horizontally so that the whole pump assembly may pivot as the plunger rod moves with the crank arm.

A glass jar, with a screw top fitted with 2 metal tubes, is inserted in the vacuum line between the pump and inoculating chamber. The jar prevents damage to the pump by acting as a trap for any liquid inoculum that might be drawn into the system by accident.

The various components are assembled in the base of a stool 14 in. high, 13 in. wide, and 17 in. long. The stool is made of $\frac{3}{4} \times \frac{3}{4}$-in. angle iron with a plywood top. The base is covered with an 18-gauge

steel sheet to protect the pump assembly from mud and dirt. The control switch and power plugs are mounted on the ends of the stool. A recessed carrying handle is fitted into the top of the stool.

PLANT PATHOLOGY LABORATORY
WINNIPEG, MANITOBA
AND
CEREAL BREEDING LABORATORY
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TOBACCO RINGSPOT VIRUS ON WATERMELON [1]

Robert J. Shepherd and F. Ben Struble [2]

SUMMARY

A mechanically transmitted virus was found constantly associated with the pimples disease of watermelons in Oklahoma. This virus was identified as a yellow strain of the tobacco ringspot virus through a study of symptomatology, host range, physical properties, cross-immunization, and serology. This virus was transmitted from watermelon to watermelon in 1 of 48 trials by the differential grasshopper (*Melonaplus differentialis* Thomas) but not in any of 45 trials with melon aphids (*Aphis gossypii* Glover). There was no evidence of transmission of this virus by watermelon seed.

Histologically, ringspot virus lesions on fruits were found to originate in the mesocarp as small necrotic areas subtended by hypertrophied parenchyma cells. Later, more extensive necrosis and hyperplasia occurred in these enlarged areas.

A thermolabile inhibitor of virus infection was demonstrated in sap from watermelon fruits and foliage.

Pimples disease of watermelon has been common in Oklahoma for a number of years. Symptoms appear primarily as small flat-topped or hemispherical intumescences that develop over the fruit surface. Often black necrotic lesions appear on the foliage. These features resemble early stages of watermelon anthracnose (caused by *Colletotrichum lagenarium* (Pass.) Ell. & Halst.). Except for fruit symptoms, the pimples disease usually is entirely masked in plants grown at high temperatures. Rosberg (9) found pimples disease in Texas to be associated with a virus that induced on tobacco and other hosts symptoms similar to those induced by tobacco ringspot virus. A tobacco ringspot virus from naturally infected watermelon was first reported by Pound (8).

Investigation of the pimples disease in Oklahoma also showed that a virus was associated with the disease. This paper deals with results of a study of pimples disease of Black Diamond watermelons in Oklahoma, with emphasis on previously unreported phases of the disease.

IDENTIFICATION OF THE VIRUS.—Twenty-four isolates of the virus from naturally infected watermelon (*Citrulus vulgaris* Schrad.) fruits were obtained from widely scattered localities in Oklahoma. Each of these isolates when inoculated into cowpea (*Vigna sinensis* Savi) caused identical symptoms. Little variation was observed in reactions on either tobacco (*Nicotiana tabacum* L.) or cucumber (*Cucumis sativus* L.) with any of the various isolates; thus, the virus appeared to be a rather constant entity. One of the isolates was selected for use in further studies.

Host range and symptomatology of the Oklahoma watermelon virus were studied in the greenhouse. To-

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[2] Formerly Graduate Assistant, and Associate Professor of Botany and Plant Pathology respectively.

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bacco, cucumber, cowpea, bean, and other hosts inoculated with the virus gave reactions typical of the tobacco ringspot virus (12). The virus was compared with Pound's (8) yellow strain of the tobacco ringspot virus from watermelon in Wisconsin; both viruses were found to cause identical symptoms on tobacco, cowpea, and cucumber. The Oklahoma isolate, like Pound's virus, showed characteristics of a yellow strain of the tobacco ringspot virus in symptoms induced on tobacco. The Oklahoma virus was pathogenic on petunia, thus differing in this respect from the virus Rosberg (9) obtained from watermelon in Texas.

Inactivation of the Oklahoma virus as determined in cucumber was as follows: heat—10 minutes at 70°C, dilution—1:100,000, longevity *in vitro*—8 days at 20°C. These properties were similar to those of the Wisconsin isolate of the tobacco ringspot virus (8) except for aging *in vitro*.

In attempts to confirm earlier evidence, and accordingly, prove the identity of the virus, cross-immunization and serological tests were employed. Havana tobacco plants that had reached the acquired tolerance stage of infection after inoculation with a green strain of the tobacco ringspot virus developed no additional symptoms after inoculation with the Oklahoma virus. Similar plants produced abundant symptoms after inoculation with either cucumber mosaic or tobacco mosaic viruses. Precipitin tests in which specific antisera to the Oklahoma virus from rabbits was used proved the virus to be serologically related to tobacco ringspot virus. Thus, the Oklahoma watermelon virus is very similar or identical to the Wisconsin virus and both are yellow strains of the tobacco ringspot virus. This substantiates the work of Pound (8) and of Rosberg (9) and provides additional evidence of the common and widespread occurrence of tobacco ringspot on watermelon.

TRANSMISSION TESTS WITH THE RINGSPOT VIRUS.—Differential grasshoppers (*Melonaplus differentialis* Thomas) and melon aphids (*Aphis gossypii* Glover) were used in attempts to transmit the ringspot virus from watermelon to watermelon. Systemically infected watermelon plants served as sources of inoculum. Because of frequent masking of disease in watermelon foliage, plants to which transmission had been attempted were held for 2 weeks and then indexed on cucumber for presence of the virus. Cucumber, variety Boston Pickling, was used for this purpose because of the difficulty encountered in transmission of the virus from watermelon foliage to other hosts. This difficulty will be discussed later. Melon aphids were maintained on caged watermelon plants. Grasshoppers were reared in the laboratory by the method of Haydak (5).

Grasshoppers were fasted 24–36 hours previous to use in transmission trials. These insects were manipulated by hand as described by Walters (11). In 48 transmission trials, only 1 plant became infected. This indicated that grasshoppers may be potential vectors but that their efficiency is probably inadequate to account for the high incidence of the virus on watermelon, since these insects are not usually prominent feeders on this crop. Walters has reported this insect as a vector of tobacco ringspot virus from tobacco to tobacco under experimental conditions.

Forty-five attempts to transmit the ringspot virus with melon aphids were unsuccessful. Smith and Brierley (10) have recently reported the green peach aphid (*Myzus persicae* Sulzer) as a probable vector of tobacco ringspot virus from gladiolus to gladiolus. Pound (8) reported negative results with the peach aphid in attempted transmissions of the ringspot virus from tobacco to cucumber and *vice versa*.

Watermelon seed appeared to be a possible means of transmission of the ringspot virus. Seed transmission was investigated with seed from naturally infected melons. All melons from which seed were saved were demonstrated to be carrying the virus. Nearly 1000 plants grown from such seed were indexed on cucumber; none proved to be infected. Of several thousand watermelon plants used in other phases of this investigation, many were known to have come from the seed of infected melons, yet in no instance were plants other than those inoculated found infected with the ringspot virus during greenhouse tests.

From the widespread prevalence of pimples of watermelon in Oklahoma, it would seem that if the virus is not commonly seed-borne, some weed or crop plant probably serves as a virus reservoir and that a relatively efficient vector transmits the virus to watermelon and from watermelon to watermelon. Attempts to recover tobacco ringspot virus from several common weeds in and adjacent to watermelon plantings have so far been unsuccessful. On the basis of the transmission tests reported in this section the prevalence of tobacco ringspot in watermelon remains unexplained.

HISTOLOGY OF VIRUS LESIONS ON WATERMELON FRUITS.—Small isolated lesions slightly elevated above the normal surface of the melon often appear on fruits infected with the ringspot virus. These symptoms remain relatively inconspicuous during the high temperatures of mid-season. With the arrival of cooler temperatures in late season, however, fruit symptoms become markedly more severe (Fig. 1). Lesions forming at this time often appear as raised irregular circles. These late-season lesions usually become necrotic and collapse. Atrophy of tissues immediately beneath such lesions often leads to considerable distortion of fruits.

Virus lesions, when cut into thin sections and viewed macroscopically, often had a tiny necrotic spot in association with a small translucent area. These were observed only in the outer cortex of fruits under raised surface lesions. The nature of these lesions on naturally infected fruits was investigated.

Representative lesions were excised throughout the growing season and preserved in formalin-acetic acid-alcohol. After dehydration in a tertiary butanol + ethanol series and embedding in paraffin, microtome

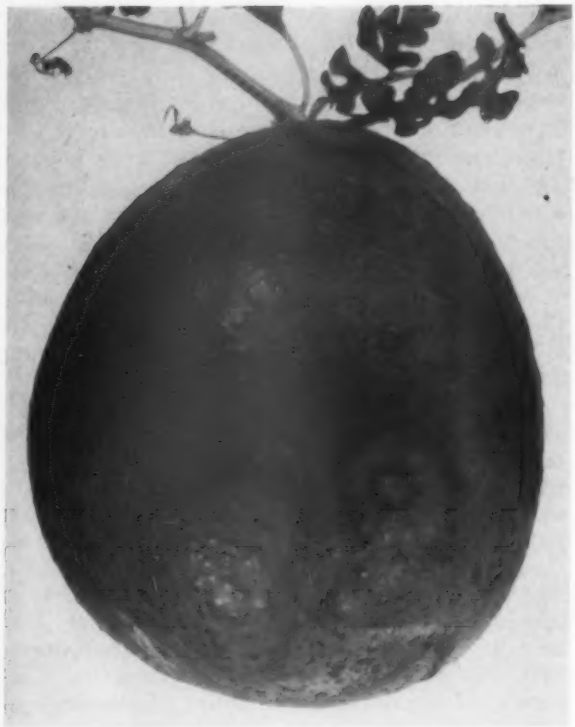

FIG. 1. Severely pimpled Black Diamond watermelon fruit infected with tobacco ringspot virus.

sections 15μ in thickness were stained with either tannic acid + ferric chloride or Flemming's triple stain.

Watermelon fruits are divided into successive layers (1), the outermost of which is epicarp and composed of a single sheet of epidermal cells. The next layer is hypoderm, which consists of several layers of cells containing numerous chloroplasts. The innermost layer is mesocarp, the outer cells of which are thick-walled and pitted. These stone cells form an almost continuous sheath around the fruit except at intervals where the sheath is broken by parenchymatous cells (Fig. 2,A). Mesocarp cells interior to the sclerenchymatous sheath are thin-walled and isodiametric. Large intercellular spaces occur throughout this loosely packed tissue (Fig. 2,A).

Microscopic observation showed that the elevated nature of ringspot virus lesions was due to small galls in the outer mesocarp of the fruit. These galls consisted primarily of hypertrophied mesocarp cells below the sclerenchymatous sheath (Fig. 2,B). A small amount of necrosis was always evident in these galls.

Hypertrophied mesocarp cells formed the bulk of most galls, although hyperplasia in varying amounts was observed in galls when considerable necrosis was present (Fig. 2,D). Hypertrophy and at least some necrosis was apparent in all galls sectioned and observed. Necrosis usually appeared as a disorganized mass of cell debris tightly compressed against the sclerenchymatous sheath and was often observed to extend into the parenchymatous rifts in the sheath (Fig. 2,B). Mesocarp cells immediately beneath and to each side of this necrotic mass were elongated in a direction perpendicular to the necrosis. During enlargement, mesocarp cells lost their normally isodiametric nature and became long and asymmetrical (Fig. 2,B). Hypertrophy resulted in a compression of tissues in galls, as large intercellular spaces normally present in this region of the mesocarp were totally absent. Compacting of these cells probably accounted for their translucent appearance when viewed with the unaided eye.

Hypertrophied mesocarp cells of lesions in later stages of development apparently had undergone several transverse divisions. Many cross-walls were visible perpendicular to the long axis of these cells, and in many cases, long tiers of these cells resulted. Such tiers invariably appeared to radiate directly inward toward the necrotic center of the gall. In lesions with considerable necrosis, particularly those developing in late season, hyperplasia of mesocarp cells closely adjacent to the necrosis resulted in the formation of a cambial-like zone of cells surrounding the necrotic center (Fig. 2,C and D). As a result, necrosis appeared to be occluded or corked out.

The ontogeny of virus lesions on watermelon fruits appeared somewhat analogous to the development of epidermal blisters on tomato fruits infected with a severe type of mosaic (3). Galls on watermelon appear to begin with the death of a few cells below the sclerenchymatous sheath. Mesocarp cells surrounding this necrosis then enlarge and become elongated toward the necrotic center. Hypertrophied cells usually were observed to form parallel columns growing in and directly toward the necrosis. This limited necrosis with subsequent hypertrophy is the final stage of development of lesions appearing during warm weather. Lesions forming during cooler periods in late season, however, develop extensive necrosis that also involves the hypodermal tissues. At the same time, hypertrophied mesocarp cells adjacent to the necrosis become meristematic and produce a zone of hyperplastic tissue around the debris of dead cells.

INHIBITION OF INFECTION BY WATERMELON SAP.—In the course of the studies reported herein, it was noted that the ringspot virus was not transmissible from systemically infected watermelon foliage to cowpea. This virus was, however, readily recovered from watermelon fruits.

These observations suggested an inhibitor of infection in ground watermelon foliage. Tests were made in an attempt to demonstrate whether or not watermelon foliage does contain such an inhibitor.

All inocula used in these tests were from systemically infected Havana tobacco. Cowpeas, variety Black, served as assay plants for inocula containing the tobacco ringspot or cucumber mosaic viruses. *Nicotiana glutinosa* L. was used to assay preparations of the tobacco mosaic virus. All watermelon extracts to be tested for inhibitive action were mixed with equivalent volumes of virus-containing sap and were

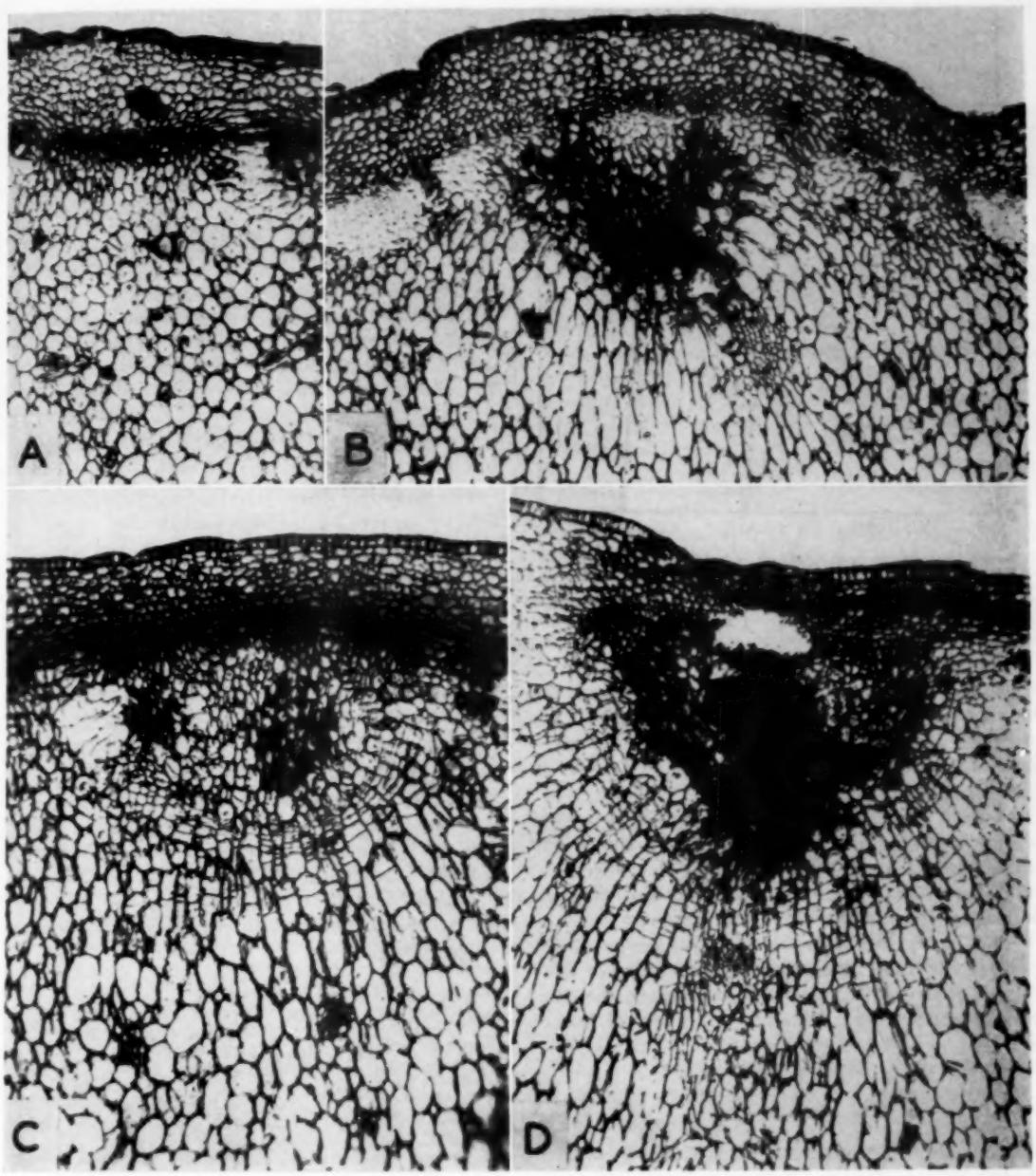

FIG. 2. Histological nature of tobacco ringspot virus lesions on Black Diamond watermelon fruits. A) Section of healthy fruit. B) Elevated lesion showing necrosis and hypertrophy of mesocarp cells. C, D) Lesions in late season showing hyperplasia of cells near necrosis. Note extensive necrosis extending through sclerenchymatous sheath and hypoderm in D.

compared with a standard inoculum by the half-leaf method. Standard inoculum consisted of virus-containing tobacco sap diluted with an equal volume of distilled water. Primary leaves of cowpea were inoculated with glass spatulas dipped in the inoculum. Cotton pads were used to inoculate half-leaves of *N. glutinosa*. All leaves were lightly and evenly dusted with Carborundum previous to inoculation.

Several tests showed that watermelon foliage sap contains a potent inhibitor of infection for the tobacco ringspot virus. Half-leaves inoculated with the standard inoculum usually produced several hundred lesions, whereas no lesions developed on half-leaves inoculated with virus-containing tobacco juice plus an equal volume of watermelon foliage sap. This demonstrated the presence of a material in ground watermelon foliage capable of totally inhibiting highly active preparations of the ringspot virus. Similar tests

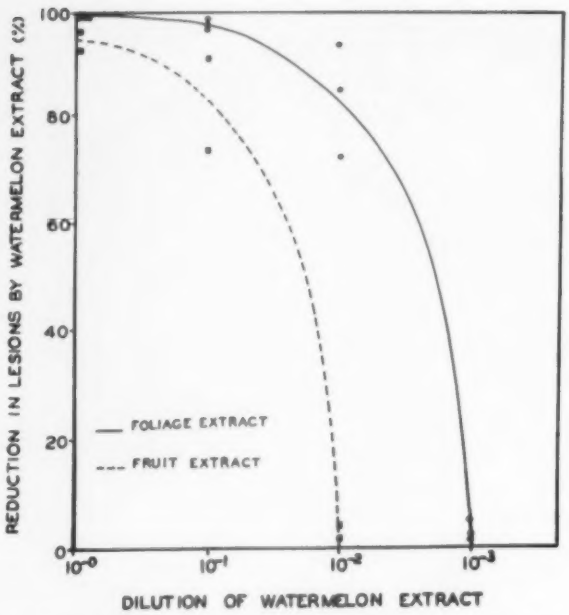

FIG. 3. Effect of dilution of the inhibitor in watermelon fruit and foliage saps in preventing infection with tobacco ringspot virus on primary leaves of cowpea.

with expressed sap from the rind areas of watermelon fruits showed a similar inhibition; however, fruit extracts were incapable of completely inhibiting highly infectious preparations of the ringspot virus from tobacco.

The effect of dilution on the inhibitive action of watermelon foliage and fruit saps was studied. The results obtained from several dilution tests were in general agreement (Fig. 3). The dilution end-point of inhibition of watermelon foliage sap was between 10^{-2} and 10^{-3}. Inhibition was, in all cases, still significant at the 10^{-2} dilution. Similar effects of dilution were observed with fruit saps, although the dilution end-point was approximately 1/10 that of foliage saps.

Watermelon foliage saps were found also to inhibit infection of *N. glutinosa* by tobacco mosaic virus and of cowpea by cucumber mosaic virus. Tobacco sap containing tobacco mosaic virus mixed equally with watermelon foliage sap gave an average of 33.6 lesions per half-leaf as compared to 244.7 lesions per half-leaf with a standard inoculum containing the same amount of virus. In no instance were tobacco mosaic virus preparations completely inhibited. With cucumber mosaic virus, however, inhibition apparently was complete when undiluted watermelon foliage sap was used.

The inhibitive action of watermelon foliage sap was found to disappear upon heating at 80°C for 10 minutes. The thermolabile nature suggests a proteinaceous material similar to that in inhibitors from pokeweed (6) and spinach (7). Bawden (2) has termed such materials "inhibitors of infection" to distinguish them from those that inhibit virus increase.

The inhibitor from watermelon was found ineffective in preventing transmission of the ringspot virus to cucumber. For this reason cucumber was the only reliable host found for indexing watermelon for presence of the ringspot virus. Gendron and Kassanis (4) have found the potency of several inhibitors of infection from higher plants to depend greatly upon the species of plant to which transmission is attempted. In their tests, cucumber was less affected by the several inhibitors used than were any of several other species. They also noted that inhibitors from higher plants usually fail in preventing infection of species from which they can be obtained. Bawden (2) has suggested that cucumber may contain the inhibitors and thus is insensitive to their action, although cucumber probably does not contain the inhibitors to the same degree as other plants, since many viruses are known to be transmitted easily from this host to other species.

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY
OKLAHOMA AGRICULTURAL EXPERIMENT STATION
STILLWATER, OKLAHOMA

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STUDIES ON THE AMINO ACID COMPOSITION AND NUTRIENT REQUIREMENTS OF THE WHEAT BUNT FUNGUS [1]

Hazel C. Murray and F. P. Zscheile

SUMMARY

The amino acid composition of *Tilletia caries* (DC.) Tul. was studied by the technique of qualitative paper chromatography. Amino acids were determined in extracts of chlamydospores before and after germination, in acid hydrolysates prepared from chlamydospores and from mycelial cultures, in aqueous media after growth of mycelial cultures, and in solutions exuded by mycelia. Twenty-one amino acids were identified in aqueous media of mycelial cultures, and many of the same amino acids occurred also in the extracts and hydrolysates of chlamydospores and mycelia. All of the amino acids which were detected were formed from L-asparagine, since this amino acid was the only source of nitrogen.

Growth rates of *T. caries* on various amino acid media demonstrated the superiority of a simple medium, containing glycine and DL-α-alanine as the source of nitrogen, over all media studied previously. Additional supplements of single amino acids and 7 other substances had little effect on growth rates. Acids of the Krebs cycle did not promote growth. L-Leucine was generally stimulatory. There was evidence of antagonistic effects among amino acids.

Little is known about the biochemical aspects of bunt resistance inherent in certain wheat varieties. A study of the nutritional requirements of *Tilletia caries* (DC.) Tul. may provide important preliminary information. Studies by Zscheile (8) demonstrated certain mineral and vitamin requirements. He showed that certain amino acids produced better growth than inorganic sources of nitrogen; of various single amino acids and combinations of these acids tried, L-asparagine alone at 0.5 M concentration proved best. Thiamine was the only vitamin shown to be of value in stimulating growth of mycelia.

The present investigation was undertaken to determine the amino acids synthesized by *T. caries* as a possible guide to the amino acid requirements of the organism. The amino acid composition was determined of the following: 1) chlamydospores, 2) germinated chlamydospores, 3) mycelium, 4) the liquid exudate from older colonies, and 5) the liquid medium following growth. The effects of other growth-promoting factors such as adenosine triphosphate, choline, guanine, pyridoxal, and uracil were investigated also. Combinations of amino acids were studied with the objective of increasing the *in vitro* growth rate of *T. caries*.

MATERIALS AND METHODS.—For preliminary work, *T. caries* (Race T-1) was first cultured for several weeks on a minimal medium MTO (8) adapted from that described by Rodenhiser and Hurd-Karrer (7). The fungus was then removed by filtration and the remaining liquid medium examined for amino acids. Since this medium contains only inorganic nitrogen, growth is very slow and there are negligible amounts of amino acids liberated in the medium. The maximal medium, MT2 of Zscheile (8), also was evaluated. Considerable amino acid synthesis and excretion occurred on this medium, which contains L-asparagine as a source of nitrogen. The large amounts of L-asparagine remaining in the medium made it difficult to identify certain other amino acids. The medium that was finally adopted was identical with the MT2 medium of Zscheile, with the exception of the L-asparagine content, which was reduced to 1/10 of that in the original.

Mycelial inoculum was obtained from cultures repeatedly transferred and grown for 2 weeks on MTO medium. In this way, a minimum of growth substances was added via the inoculum. The fungus and culture media were ground together in a small homogenizer; the resulting inoculum was then added to flasks of liquid media aseptically by means of a syringe. Cultures were seeded with 3–6 drops of inoculum and grown for 2 weeks at 20°C. At the end of this time, mycelia were removed by filtration. The remaining liquid was autoclaved 15 minutes to destroy enzymes and then shaken with resin (Rohm and Haas IR 120, a cation exchanger) for 1 hour to remove cationic amino acids from extraneous substances (A. L. Levy, personal communication, 1954). The resin was treated with 10 per cent ammonia, which eluted the acids. The eluate was evaporated to dryness on a steam bath and made to volume.

The amino acid composition of an alcoholic extract and of an acid hydrolysate of chlamydospores was determined. The extract was obtained by refluxing ground bunt balls of *T. caries* with 50 per cent ethanol for an hour. The amino acids were removed from the extract by treatment with resin as described above. Hydrolysates were made by refluxing 2 ground bunt balls with 20 per cent HCl for 24 hours, evaporating to dryness on a steam bath to remove the acid, and subsequent recovery of the amino acids with resin. Aqueous extracts of germinated chlamydospores were obtained from spores after 5 days' germination on water agar slants at 20°C. The amino acid composition of acid hydrolysates of mycelia was also determined.

Amino acids were identified by 2-dimensional paper

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chromatography (1). Chromatograms were run on Whatman No. 1 filter paper; a 4:1:5 mixture of n-butanol : acetic acid : water (BAH) was used for the first dimension and an 85:15 mixture of phenol : water for the second. Chromatograms were sprayed with 2 per cent ninhydrin in n-butanol saturated with water; they then were dried at 65° C for 20 minutes. Visual examination was completed within 1 hour.

The identity of spots on chromatograms was established by comparing the R_f values of the ninhydrin-reacting spots from experimental material with those obtained from known mixtures, as well as by superposing known amino acids over spots of experimental material before irrigating the chromatograms with solvent.

All chromatograms were rated qualitatively by visual inspection according to size and intensity of spots. Visual estimation of spots formed in the ninhydrin reaction had to be qualified by some knowledge of the relative sensitivities of the various amino acids with respect to color development. The tables given by Moore and Stein (5) were useful in this regard. An indefinite spot near the point of origin of many chromatograms was assumed to be cystine. Since this acid chromatographs so poorly, its identification was doubtful.

For the nutritional study, the 3 basic media used contained the following: 1) DL-α-alanine, DL-aspartic acid, L-glutamic acid, glycine, and DL-serine; 2) DL-α-alanine, DL-aspartic acid, and glycine; and 3) DL-α-alanine and glycine. All were used in M/10 total amino acid concentration. All contained thiamine, sucrose, and minerals as in the MT2 medium. When supplemental amino acids were employed, 1 ml (M/10) was added to each flask containing 15 ml of media. When other supplements were employed, they were added to each flask in the following amounts: adenosine triphosphate, 5 mg; choline, 1 ml (M/20); ethanolamine, 1 ml (M/10); 3-indole acetic acid, 50 gamma; pyridoxal, 9 gamma; guanine and uracil, 10 mg. The pH was adjusted, when necessary, to 6.5–7.0 before autoclaving. Measurements of pH of the medium following growth of the fungus were made at intervals throughout the studies, and no appreciable changes were found. This further confirms a former conclusion (8) that thiamine prevents development of acidity in such cultures. For most experiments, 50-ml Erlenmeyer flasks were employed in triplicate. Experiments were replicated 3 or more times. Cultures were grown for 2 weeks at 20°C, after which the mycelium from 3 flasks was removed, washed, dried, and weighed.

RESULTS.—The amino acid composition of ethanol extracts of chlamydospores was similar to that of the hydrolysates, with the exceptions that serine was lacking in the extracts and ornithine was absent from the hydrolysates (Table 1). The hydrolysates of chlamydospores did not contain ethanolamine, histidine, or

TABLE 1.—*Relative amino acid contents of chlamydospores and exudate of T. caries and of media after growth*

Amino acid	Chlamydospores		Aqueous extracts of germinated chlamydospores	Acid hydrolysates of mycelium	Exudates	Aqueous media
	Ethanol extracts	Acid hydrolysates				
α-Alanine	+++[a]	+++	+++	+++	+++	+++
Arginine	+++	++	++	++	+	++
Asparagine					+++	+++
Aspartic acid	+++	+++	+++	+++	+++	+++
Cystine						++
Djenkolic acid	++	+	++		++	++
ε-Amino caproic						+
Ethanolamine			+	+		+
γ-Amino butyric acid	+++	++	+++	+	+	++
Glutamic acid	+++	+++	+++	+++	+++	+++
Glutamine			+		++	+
Glycine	+++	+++	+++	+++	++	++
Histidine				++		++
Leucine	++	++	+	++	++	+++
Lysine	+++	++	++	++	++	++
Ornithine	+		+	+		+
Phenylalanine	+	+	+	+	+	+
Proline	+	+	++	++	+	+
Serine		+++	+++	+++	++	+
Threonine	+	++	+	++	+	+
Tyrosine	+	+	+	+	+	+
Valine	++	++	++	++	++	+++
Unknowns	+[b]		+[b]		+[b, c]	+[b, c]

[a] The number of + signs indicates the relative amounts of each amino acid, as estimated by visual examination of paper chromatograms.

[b] Pink spot; movement in BAH equals that of proline; movement in phenol-water slightly greater than that of proline.

[c] Two pink spots near origin.

ornithine but did contain djenkolic acid in small amounts. When chlamydospores were germinated, ethanolamine, glutamine, and serine appeared in addition to the amino acids found in an alcoholic extract of the nongerminated spores.

Acid hydrolysates of the mycelium contained no ε-amino-caproic acid or djenkolic acid. Since cystine is destroyed by acid digestion, this may have accounted for its absence. Glutamine and asparagine would, of course, be converted to glutamic and aspartic acids, respectively, by this treatment. α-Alanine, aspartic acid, glutamic acid, glycine, and serine were most abundant in the acid hydrolysates.

The exudates of mycelial cultures rarely appeared except on media containing L-asparagine. The amino acid concentration of exudates was high, and chromatograms could be made from them directly. The exudates contained djenkolic acid but not ethanolamine or ornithine, substances that were present in the medium and in acid hydrolysates of mycelia.

More amino acids were present in the aqueous medium after growth of the fungus than in any other material chromatographed. ε-Amino caproic acid was present in addition to the acids that were found previously. Cultures supplied with L-leucine develop a striking red coloration in the medium within 2 weeks. Later such media become brown, similar to older cultures in MT2.

The large amounts and general occurrence of α-alanine, aspartic acid, glutamic acid, glycine, and serine in fungal material suggested that they should be used in a basic mixture of amino acids in studies on the effects of additional single substances on the growth of *T. caries;* therefore, these materials were used in such studies (Table 2). Three, and finally 2, amino acids were employed later. In many instances the 2 amino acids, glycine and DL-α-alanine, served equally as well as, and possibly better than, more complex mixtures.

Most of the supplementary substances (Table 2) were physiologically inactive, as measured by growth under these conditions. A few substances retarded growth, as shown in preliminary experiments. Despite the apparent presence of cystine in preparations from *T. caries*, cystine or methionine severely inhibited growth when added as a supplement. ε-Amino caproic acid also retarded growth when added to the glycine-α-alanine basic mixture in a single experiment.

Some acids of the Krebs cycle also were studied for their effects on growth of *T. caries.* To each flask containing 25 ml of medium, 0.25 ml of M/10 acid was added to MT2 or to MTO plus thiamine (25γ per 25 ml of medium). No appreciable change in growth rate occurred when aconitic, α-ketoglutaric, fumaric, malic, oxalacetic, pyruvic, or succinic acid was added to the medium. Citric acid always depressed growth.

DISCUSSION.—A limited number of reports on amino acid composition of fungi appear in the literature. Broyles (2) reported qualitative and quantitative differences in the amino acid composition of urediospores among races of *Puccinia.* Ethanol extracts of urediospores contained the amino acids reported in Table 1 for chlamydospores, but lacked djenkolic acid, ornithine, and proline. In addition, his extracts contained asparagine, cystine, glutamine, histidine, methionine, serine, and 11 unidentified ninhydrin-reacting compounds. Upon hydrolysis, proline and homocystine were found. In the present study when chlamydospores of *T. caries* were hydrolyzed, only serine appeared as an additional amino acid. Thus the amino acid compositions of spores from these 2 fungi differ considerably.

Powell and Strange (6) demonstrated certain biochemical changes that occur during the germination of bacterial spores of *Bacillus megatherium* de By. and *B. subtilis* (Cohn) Prazmowski. By paper chromatography, they showed that these spores on germination excrete alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, serine, tyrosine, and valine. The exudate on hydrolysis also yielded threonine, α-ε-diaminopimelic acid, and glucosamine.

DeVay (4) demonstrated by paper chromatography the presence of alanine, arginine, aspartic acid, γ-

TABLE 2.—*Comparative growth rates of T. caries on media with different amino acids as sources of nitrogen and with different added nutrients*

Medium	Mg dry weight[a] following growth for 2 weeks at 20°C in media containing indicated combinations of amino acids			
	5 Amino acids[b]	3 Amino acids[c]	2 Amino acids[d]	
	in 50-ml flasks	in 50-ml flasks	50-ml flasks	125-ml flasks
MT2 (control)	126± 8	146± 5	138±15	141±18
Basic mixture	149±12	153±14	183±17	262±22
Basic mixture plus				
L-Arginine			259±26	199±22
γ-Amino butyric acid	183±29	158±23	189±31	
L-Histidine	93±14			241±52
L-Leucine	195±29	202±11	258±35	312±22
DL-Lysine	152±18	174±18	168±22	
DL-Ornithine			255± 4	238± 8
DL-Phenylalanine	155±12	186± 8		
DL-Pipecolic acid		191±22		186±10
L-Proline	174±29	168±11	179±24	
DL-Serine		174±13	202±25	262±42
DL-Threonine	141±18	167±14	207±33	252±41
DL-Valine	159±26		193±32	
Adenosine triphosphate	159±10	180±10	252± 3	270±19
Choline	156±11	144±16	226±20	260±26
Ethanolamine		162±13	203±23	206±18
Guanine				314±69
Indole acetic acid	152± 6	156±25		
Pyridoxal	168± 7	130± 7	174±13	
Uracil				344±63

[a] Dry weights are given as averages (of 3 experiments) with the standard errors of the means.
[b] Glycine, DL-α-alanine, DL-aspartic acid, DL-serine, and L-glutamic acid.
[c] Glycine, DL-α-alanine, and DL-aspartic acid.
[d] Glycine and DL-α-alanine.

amino butyric acid, glutamic acid, glutamine, glycine, leucine, lysine, phenylalanine, proline, serine, tyrosine, and valine in ethanol (70 per cent) extracts of culture of *Ustilago zeae* (Schw.) Ung. Nitrogen was supplied as an inorganic source. When hydrolysates of the mycelium were chromatographed, cystine, methionine, histidine, and 2 unknowns were found in addition. The capacity of *T. caries* to synthesize amino acids appears to be somewhat comparable to that of *U. zeae*, with the exception that *T. caries* also synthesizes djenkolic and ϵ-amino caproic acids. Methionine was never found in any preparation from *T. caries*.

Converse (3) found that the barley stripe fungus, *Helminthosporium gramineum* Rabh., produced more mycelial growth on media in which certain amino acids supplied part of the nitrogen; glycine was one of the more useful amino acids in this regard.

It is obvious that *T. caries* can synthesize a complex variety of amino acids from the 2- and 3-carbon amino acids (glycine, DL-α-alanine, and carbon chains from sucrose). So far as vegetative growth is concerned, *T. caries* is a fairly independent, though slow-growing, parasitic fungus. This is further illustrated by the fact that so few of the substances listed in Table 2 caused an appreciable increase in growth. Development of chlamydospores was not observed in these experiments.

Zscheile (8) reported earlier that L-leucine did not encourage growth of *T. caries* in media similar to those employed in the present study. In contrast, in the present work but employing different combinations of amino acids and inorganic nitrogen, L-leucine was more stimulatory generally than any other single substance that was evaluated. As shown in Table 2, some nutrients promote growth when added to one basic medium but may have little or no effect when added to a medium containing another combination of amino acids. These effects may be due to antagonistic influences exerted by some amino acids on others. Guanine and uracil also appeared to stimulate growth when added to basic media.

The well-developed ability of *T. caries* to synthesize amino acids makes it unlikely that any of the amino acids considered here would be limiting factors in the production of chlamydospores.

DEPARTMENT OF AGRONOMY
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA

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LISTING OF BOOKS

PHYTOPATHOLOGY will, from time to time as space becomes available, list titles of books on pathological subjects. A brief description of the contents of the books will be included.

HAUPT, A. W. 1956. **An introduction to botany.** Ed. 3. McGraw-Hill Book Co., Inc. New York, Toronto, London. 447 p. Price $5.50.

This college textbook is designed to acquaint the reader with the basic facts and principles of plant life. Over half of the book is devoted to structure, functions, and life relations of seed plants. The remainder consists of a treatment of the major plant groups, from the algae to the seed plants. A 19-page chapter on the fungi is included. The book ends with a 20-page glossary.

HIRATSUKA, NAOHIDE. 1955. **Uredinological studies.** Kasai Publishing Co. Tokyo, Japan. (In Japanese) 382 p., 79 Figs., X Pl. Available from the author at $10.00 per copy.

A full treatment of the rust fungi for Japanese readers with an up-to-date bibliography of 839 items. As all aspects of rust investigations are here treated by a distinguished student of the rusts, it seems regrettable that the book is not available in English.

EXTENT OF CROSS PROTECTION AMONG STRAINS OF CUCUMBER MOSAIC VIRUS IN CUCUMBER AND COWPEA[1]

J. B. Sinclair and J. C. Walker

SUMMARY

The extent to which cross protection among strains of cucumber mosaic virus (CMV) occurs in cucumber (*Cucumis sativus* L.) and cowpea (*Vigna sinensis* Savi.) was studied in the greenhouse and field. In field experiments in 1952, 1953, and 1954, cross protection between strains of the virus was not complete, presumably because of a failure of any 1 strain to become thoroughly established in cucumber, thus affording areas of susceptibility for other strains. Greenhouse experiments showed that at least 2 strains of CMV could multiply simultaneously in the same cucumber plant and that complete cross protection between strains did not occur. Primary leaves of cowpea inoculated with strain 14 of CMV offered better cross protection against strains that produced local lesions than did systemically infected trifoliate leaves, but the former tended to drop after the second inoculation. No inhibition or protection was noted when inoculation of primary leaves with a local-lesion strain was followed by either a second inoculation on the trifoliate leaves with the same virus or by an inoculation of primary leaves with strain 14.

The type of immunity exemplified by an experiment described by Thung (5), wherein tobacco plants inoculated with a strain of tobacco mosaic virus (TMV) were resistant to a second strain of the same virus, is commonly referred to as cross protection. He believed that only 1 virus strain could occupy a given plant cell and only a single virus strain could multiply in a given cell.

A similar type of immunity was found by Price (2) to exist between strains of cucumber mosaic virus (CMV) on zinnia. All described strains of CMV produce mottle symptoms on zinnia (*Zinnia elegans* Jacq.) except Price's No. 6 strain (P6), which induces local lesions. Zinnia plants systemically infected with a mottle-producing strain fail to develop local lesions when inoculated with the P6 strain. This test has been used widely as a criterion of strain relationships in CMV.

Experiments in the greenhouse and field were conducted to determine to what extent cross protection occurred between strains of CMV in cucumber (*Cucumis sativus* L.) and cowpea (*Vigna sinensis* Savi.). It was of interest to determine whether a mild strain of CMV infecting cucumber in the field would protect against a more severe strain. If such were the case, many kinds of field tests with different strains could be carried on in adjacent plots.

VIRUS STRAINS AND ASSAY HOSTS.—Three strains of CMV that were easily identified on zinnia and Black cowpea were used. Strain Y produces a green mottle on zinnia and local lesions on cowpea. It was originally isolated from spinach in Wisconsin (1) and is regarded as typical of the usual wild strain collected on naturally infected cucurbits in the Madison, Wisconsin, area. Strain 14 also produces a green mottle on zinnia but infects cowpea systemically. This strain was isolated from bean at Madison by Whipple and Walker (6). Price's No. 6 strain produces local lesions on zinnia and cowpea (2). By use of these assay hosts and virus strains, it was possible to distinguish the type of virus present in inoculated cucumber plants.

CROSS PROTECTION IN CUCUMBER.—In 1952, a series of randomized replicate blocks were planted with National Pickling (NP), a mosaic-susceptible variety, in the field. Plants in different blocks were inoculated in the third leaf stage with various strains of CMV, including P6 and Y. At the end of the season, 25 mottled fruits were collected at random from those rows containing plants inoculated with Y and from rows containing plants inoculated with P6. Juice was extracted from each fruit separately and used for the mechanical inoculation of zinnia. All assays from fruits from Y rows gave typical systemic mottle. All assays from fruits from P6 rows gave systemic mottle similar to that incited by Y. It thus appeared that cross inoculation had occurred generally during the season and that strain Y had replaced P6 in the plants inoculated with the latter. The possibility arose that P6 had mutated during the season to a strain systemic in zinnia, but this seemed unlikely because the systemic mottle on zinnia was of the green type and not of the yellow type that appears when P6 mutates to a systemic strain on zinnia in the greenhouse. The results indicated strongly that the P6 strain did not protect against strain Y or other strains of CMV and that during the season P6 was completely eliminated.

In 1953, the varieties NP and Ohio MR17 (MR17) were inoculated in the field with strains Y, P6 and 14. MR17 is resistant to CMV, but the virus can multiply in this variety once infection is established. Plants were inoculated in the third-leaf stage, and assays were made at various intervals during the growing season. Since the CMV inhibitor in cucumber tissue

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extract is absent in the flower petals or is present in low concentrations (4), assays were made with petal tissue. Blossoms were collected and assayed separately from each replicate.

For some reason not explained, the assay inoculations at 29 days and at 65 days after inoculation incited symptoms on cowpea but usually not on zinnia, whereas at 81 days both assay hosts generally were infected (Table 1). An examination of the data shows that there was, as in 1952, no evidence of general cross protection. In no case was P6 recovered from plants originally inoculated with P6. Instead, strains Y and 14, either singly or in combination, were obtained from all such plants. There was also no consistent protection between Y and 14. In most of the plants tested at 81 days, the original strain was partially or wholly replaced by the other. All of the noninoculated plants in the buffer row became infected by Y or by a wild strain similar to Y.

In 1954, the 3 viruses were used for the inoculation of NP grown in 25-ft. rows that were in random order in 3 replicated blocks. The plants were in the third-leaf stage when inoculated. Other varieties in the blocks, which included MR17, were inoculated at the same time with strain Y only. In such an arrangement, each virus strain had equal opportunity, on the average, to be transmitted naturally to each of the other types of inoculated plants. Two plantings were made, 1 on May 19 (comparable to the 1952 and 1953 planting dates) and 1 about 5 weeks later on June 25. Petals were collected at random from NP at various intervals from all replicates of a virus-variety combination and combined for a single assay. In the first planting, assays from plants inoculated with P6 showed only the presence of that strain at 28 and 35 days; at 59 and 74 days there was no evidence of P6, whereas Y and 14 were both found (Table 2). In assays made throughout the experiment on Y rows, only strain Y was recovered. Assays on strain 14 rows showed the presence of P6 at 59 days and of P6 and Y, but not 14, at 74 days. The fact that strain 14 could not be recovered from plants inoculated with that strain but could be recovered from other plants in which it was present in combination with other strains could not be explained. In the late planting, where high temperatures followed immediately after inoculation, the plants in P6-inoculated rows were severely dwarfed and most of them died within 2 weeks. The plants in strain 14 rows were dwarfed promptly. Those in the Y rows did not show mottle until about 3 weeks after inoculation. The assays of P6 rows were therefore from relatively few plants. In the P6 rows, P6 was present at 26 days but at no time later; 14 was present at 33 days; and both 14 and Y were present at 54 days. In the Y rows, the Y strain was the only one identified, but assays were not made after 54 days. In the strain 14 rows, P6 was found exclusively at 33 and 39 days; Y and P6 were found at 54 days and Y and 14 at 61 days.

It was evident from the field assays that strains 14 and P6 could be recovered from the blossoms of cucumber, but no data have been obtained as to whether

TABLE 1.—*Reactions of assay hosts to inoculations from National Pickling (NP) and Ohio MR17 (MR17) varieties of cucumber previously inoculated in the field in 1953 with different strains of CMV*

Replicate	Variety and virus	Reaction[a] of assay hosts in tests made at indicated number of days after inoculation					
		29 days		65 days		81 days	
		Zinnia	Cowpea	Zinnia	Cowpea	Zinnia	Cowpea
I	NP-Y	O	L	S	L	S	O
	NP-P6	O	L	O	L	S	L, S
	NP-14	O	L	O	L	S	S
	MR17-Y	O	L	O	L	S	S
	MR17-P6	O	L	O	L	S	L, S
	MR17-14	O	L	O	L	S	L
II	NP-Y	O	L, S	O	L, S	S	L, S
	NP-P6	O	L	O	L	S	S
	NP-14	S	L, S	O	L, S	S	L, S
	MR17-Y	S	L	O	L	S	L
	MR17-P6	O	L	O	L	S	L
	MR17-14	O	L	O	L, S	S	S
III	NP-Y	O	L	S	L	O	L, S
	NP-P6	O	L	O	L, S	S	L, S
	NP-14	O	L, S	S	L	S	S
	MR17-Y	O	L	O	L	S	L, S
	MR17-P6	O	L	O	L	O	L
	MR17-14	S	L	O	L, S	S	L, S
Buffer (noninoculated)	NP	O	L	O	L	S	L

[a] L = local lesions; S = systemic mottle; O = no symptoms. Presence of strain P6 indicated by local lesions only on zinnia and cowpea, that of Y by local lesions on cowpea and systemic mottle on zinnia, and that of 14 by systemic mottle in zinnia and cowpea.

TABLE 2.—*Reactions of assay hosts to inoculations from National Pickling cucumber planted at dates indicated in the field in 1954 and inoculated with strains Y, P6, or 14 of CMV*

Days after inoculation	Reaction[a] of zinnia and cowpea to inoculations from rows originally inoculated with virus indicated											
	Planting of May 19						Planting of June 25					
	Zinnia			Cowpea			Zinnia			Cowpea		
	Y	P6	14	Y	P6	14	Y	P6	14	Y	P6	14
26							O	L	O	L	L	S
28		L		L		L						
33							S	O	L	L	S	L
35		L		L	O	O						
39							S	S	L	L	O	L
46	S	O	O	L	O	L						
53	O	O	O	L	O	L						
54							S	S	L,S	L	L,S	L
59	S	S	L	L	L,S	L						
61									S			L,S
74	S	S	L,S	L	L,S	L						

[a] L = local lesions; S = systemic mottle; O = no symptoms. Presence of strain P6 indicated by local lesions only on zinnia and cowpea, that of Y by local lesions on cowpea and systemic mottle on zinnia, and that of 14 by systemic mottle in zinnia and cowpea.

or not these strains can be obtained more readily from corolla than from leaf tissue as was reported with experiments with strain Y (4).

Additional studies were made in the greenhouse where insect vectors and routine field handling were eliminated. Before greenhouse experimentation began, it was desirable to know the minimum time required for CMV to invade young cucumber plants systemically, since it may be assumed that all cells of the host must be invaded by the virus if complete cross protection is to be accomplished. In 4 experiments, NP plants in the 3- to 5-leaf stage were inoculated on cotyledons with Y. At intervals following this inoculation, tip leaves from 6 plants were assayed on cowpea. Each experiment consisted of at least 10 assay plants. In only 1 experiment was virus recovered in 48 hours and then only 2 of 10 cowpea plants inoculated became infected. In the 3 additional experiments, 50–100 per cent of the assay plants showed that CMV had become established in growing tips of young cucumber at 96 and 144 hours after inoculation. It was apparent that under the conditions used, a minimum of 96 hours were required for Y strain of CMV to move from inoculated cotyledons to tip leaves of cucumber. If cross-protection tests were to be made with such plants, the second inoculation should not be made until at least 8 days after the first.

Three experiments were then conducted in which 12 cucumber plants in the 2- to 3-leaf stage were inoculated with Y, P6, or 14. Two weeks later, when systemic symptoms were evident and ample time had been given for thorough infection, 6 plants of each series were reinoculated with 1 of the other 2 strains. At the beginning of the experiments, another 6 plants were inoculated with a mixture of 2 of the 3 strains in various combinations. MR17 plants were used in the first experiment. Assays were run at different time intervals on cowpea and zinnia, 1 or both assay hosts being inoculated for any single assay depending on the virus or virus combination being tested (Table 3). Plants originally inoculated with Y, P6, or 14 yielded only the original virus throughout the experiment, indicating that the original strain was not displaced by another under these conditions. From plants inoculated simultaneously with Y and P6, only Y was recovered in assays made after 60 days or less. Both viruses were identified on the 90th day. From plants inoculated simultaneously with P6 and 14, both viruses were recovered on the 60th day but only P6 was on the 90th day. When Y was followed 2 weeks later by P6, both viruses were identified at 25, 32, and 90 days, but not at 60 days. When P6 was followed by Y, both viruses were recovered at 32 days but only P6 at 60 and 90 days. When P6 was followed by 14, only P6 was recovered at 25 days, but both were recovered in later assays. Two additional experiments were conducted in which NP variety was used, and assays were made before and after the second inoculation. Confirmatory results were obtained in both cases.

Data from 3 greenhouse experiments show that cross protection was not complete in either resistant

TABLE 3.—*Reactions of assay hosts to inoculations from Ohio MR17 cucumber plants inoculated in the greenhouse with strains of CMV, either singly or in pairs used simultaneously or at a 2-week interval*

Strain used for indicated inoculation		Reaction[a] of assay hosts to assay inoculations made at indicated intervals after original inoculation					
		25 days	32 days	60 days		90 days	
Original	2nd	Zinnia	Zinnia	Zinnia	Cowpea	Zinnia	Cowpea
Y			S	S		S	
P6		L	L	L		L	
14		S	S	S	S	S	S
Y + P6		S	S	S		L,S	
P6 + 14			L,S	L	L,S	L	L
Y	P6	L,S	L,S	S		L,S	
P6	Y		L,S	L		L	
P6	14	L	L,S	L,S	L,S	L,S	L

[a] L = local lesion reaction; S = systemic reaction. Presence of strain P6 indicated by local lesions only on zinnia and cowpea, that of Y by local lesions on cowpea and systemic mottle on zinnia, and that of 14 by systemic mottle in zinnia and cowpea.

or susceptible cucumber, an observation in accord with results of field experiments from 1952 to 1954, inclusive. In the greenhouse, P6 survived in plants for 90 days when preceded by Y or when used alone. In this respect, P6 reacted differently than in the field where it disappeared promptly.

CROSS PROTECTION IN COWPEA.—Cowpea is commonly used as a local-lesion host for CMV. Most strains of the virus produce discrete lesions on primary leaves of the plant; however, strain 14 invades the cowpea systemically without producing local lesions. Since P6 is a relatively unstable strain from which strains frequently arise that induce yellow systemic mottle on zinnia, it is difficult to maintain in a condition suitable for cross-protection tests. In contrast, strain 14 is very stable. Consequently, the possibility of using this strain in cowpea for determining relationships among strains of CMV was investigated.

Twelve or 14 cowpea seedlings were grown until the unifoliate leaf had partially expanded. Half of the plants were inoculated with strain 14. Typical mottle symptoms appeared 3–5 days later on the inoculated primary leaves, as well as on the expanding trifoliate leaves. Four experiments were run in which the primary leaves of the previously inoculated plants and of healthy plants were inoculated with 1 of 3 local-lesion strains. In addition to strain Y, 2 other local-lesion strains were used. In no case did local lesions appear on leaves previously inoculated with 14, whereas numerous local lesions appeared on those not previously inoculated. These tests indicated that cross protection between 14 and the strains tested did occur in the primary leaves of cowpea. Since the plants had previously been inoculated, it was possible that the inhibition of the second strains might have resulted from mechanical injury rather than from true cross protection. Unfortunately, the primary leaves thus twice inoculated often dropped before local lesions appeared on the control plants.

Experiments were next run to determine whether systemically infected trifoliate leaves could be used in cross-protection tests. In each of 4 trials, 12–14 seedling plants were inoculated on the primary leaves with strain 14. An equal number of plants were left noninoculated. When the first trifoliate leaves were nearly expanded, they were inoculated with a local-lesion strain. In all cases, numerous local lesions appeared on previously noninoculated plants. Single lesions occasionally appeared on leaflets of plants previously inoculated with 14. The dilution of the inoculum was 1:1. Better results might have been obtained if a 1:10 or a 1:100 dilution had been used.

Two additional experiments were conducted to determine whether inoculation of primary leaves of cowpea with a local-lesion-producing strain, such as Y, influences the production of local lesions on the trifoliate leaves of the inoculated plants or inhibits invasion by a systemic strain, such as 14, introduced on the primary leaves at a later date. If any inhibition or protection did occur, it would indicate that Y could invade cowpea systemically without producing obvious symptoms. No such inhibition or protection was noted in either experiment.

It may be concluded from these experiments that trifoliate leaves of cowpea systemically infected with strain 14 offered nearly as complete cross protection against local-lesion strains as did the unifoliate leaves. They served as better leaves for assay purposes in this case because of the tendency of inoculated unifoliate leaves to drop prematurely.

DISCUSSION. — An ideal host-virus relationship for cross-protection tests is offered by zinnia and CMV. The P6 strain may be considered as virulent since it produces necrotic lesions on zinnia, whereas all other less virulent strains of the virus produce systemic mottle. Price (2) has shown that inoculation of zinnia plants with mild strains will prevent the establishment of the virulent P6. It has been claimed by Thung (5) and Salaman (3) that for such complete protection all cells of the host exposed to the second virus must be thoroughly infected by the first. Such would appear to be the case in zinnia. A similar situation was shown to occur on the primary leaves of cowpea. When strain 14 of CMV was allowed to become thoroughly established on the unifoliate leaves, subsequently introduced local-lesion strains did not become established.

Although cross protection can occur between strains of CMV on 2 unrelated hosts, it is not valid to assume that it would occur on a third host. The lack of cross protection in field-grown cucumber may have resulted from failure of any one of the virus strains to become thoroughly established in cucumber plants, thus affording areas of susceptibility for other strains. In the 1953 field experiments, strain 14 became established in plants inoculated with Y and *vice versa*. The 1954 field data indicate that Y is easily established and tends to multiply at a faster rate than does 14. Greenhouse experiments confirmed field results. They also gave evidence that when 2 strains of CMV were introduced simultaneously in the cucumber plant, both could multiply simultaneously, although in many cases both did not persist until the end of the experiment. In the controls, there was no change in strains in singly inoculated plants. The fact that an occasional lesion was produced by local-lesion strains of CMV on trifoliate leaves of cowpea systemically infected with strain 14 suggests that all cells of such leaves were not infected by strain 14.

DEPARTMENT OF PLANT PATHOLOGY
UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

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INHERITANCE OF RESISTANCE TO TOBACCO-ETCH VIRUS IN CAPSICUM FRUTESCENS AND IN CAPSICUM ANNUUM[1]

W. H. Greenleaf [2]

SUMMARY

Resistance to the tobacco-etch virus in *Capsicum frutescens* L., Plant Introduction 152225 from Peru, and in *C. annuum* L. var. Cayenne, South Carolina 46252, was inherited monofactorially and determined by recessive genes. These 2 genes have been assigned the symbols et^f and et^s, the superscripts denoting their respective species origin. The corresponding dominant alleles have been designated by the symbols et^{f+} and et^{s+}. It is not known whether the genes et^f and et^s are alleles. These genes are postulated to determine a slow rate of multiplication of the virus and masking of symptoms, thus permitting a sharp separation of susceptible from resistant plants 3 weeks after inoculation.

In *C. annuum* (S.C. 46252), one or more modifying genes that increase the resistance level to apparent immunity are postulated to account for the occurrence of such plants in F_2 and among the selfed progeny of highly resistant parent plants. Whereas absolute immunity in S. S. 46252 seems improbable, the possibility of its attainment by selection of minor genes for resistance cannot be excluded.

Of the 2 resistances, that of S. C. 46252 is probably the higher, and therefore the better for use in breeding. Moreover, the sterility barrier that was encountered in the interspecific cross *C. frutescens* F_2 (P.I. 152225 × Tabasco) × *C. annuum* var. Pimiento is a deterrent to the use of P. I. 152225 for breeding varieties of *C. annuum* resistant to tobacco-etch virus. Most of the commercial pepper varieties that are grown in the United States fall in the *C. annuum* group.

In a breeding program to incorporate disease resistance in the pimiento pepper, the heritability and the relative value for breeding of 2 resistances to the tobacco-etch virus (TEV), *Marmor erodens* var. *severum* Holmes, were investigated. The first, in the Peruvian introduction *Capsicum frutescens* L., Plant Introduction 152225 (henceforth referred to as P. I.), was reported by S. G. Younkin, Campbell Soup Company, Riverton, N. J., in personal correspondence with the author in 1946; the second, in *C. annuum* L. var. Cayenne, South Carolina 46252 (henceforth referred to as S. C.), was reported by H. H. McKinney (5) in 1952. Younkin found that P. I. had remained symptomless following inoculation, whereas McKinney reported S. C. to be immune.

Subsequently, Greenleaf (1) confirmed Younkin's results but showed further that the resistance in question was that of a masked carrier rather than immunity.

Results of inoculation tests with 28 phenotypically uniform plants of S. C. grown from seed furnished the author by McKinney contradicted his results, since half of the plants promptly developed a pronounced mottle and most of the other half developed symptoms within 30 days. Several plants remained free from infection, however, even after a second inoculation, as shown by subinoculation tests to the sensitive Tabasco tester. Apparently, these plants were immune (1). Two such selections were used in the genetic analysis of resistance of this species.

P. I., a distinctive, colorful form of *C. frutescens*, has been repeatedly grown since 1945. It has a bushy compact habit, woolly leaves and stems, and pendent fruits that are varicolored, yellow-purple, or scarlet before turning a uniform red on ripening. Earlier inoculations of P. I. with TEV (1) had shown no visible variability in resistance among individual plants; hence, a relatively uniform genotype with regard to TEV resistance, suitable for genetic analysis, was available in this species. Four plants of P. I. entered the analysis of this species.

METHODS. — Three tests were conducted in a screened glasshouse during the cooler months of the year when temperatures for virus tests were most favorable. In the first test with P. I., the highly susceptible Tabasco pepper was the contrasting parent.

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[2] Vegetable Breeder, Alabama Agricultural Experiment Station, Auburn, Alabama.

In the other 2 tests, both with S. C., selections of Truhart Pimiento or of pimiento-type breeding lines served this purpose. In all experiments, populations were included of 1) the resistant parent, 2) the susceptible parent, 3) the F_1 hybrid, 4) the backcross to the resistant parent, 5) the backcross to the susceptible parent, and 6) the F_2. Seed lots of the same F_2 or backcross population, genetically similar but having 1 or more different selections in their pedigree, were kept separate. Some seed lots of different harvests, although of identical parentage, also were kept separate. The populations mentioned were further subdivided into individual flat-row samples, each from 16 seeds, to facilitate the counting and the classifying of plants into symptom classes and also to obtain many small rather than a few large samples of each population for the Chi-square (X^2) tests of the ratios.

The seeds, sown in steam-sterilized soil, were spaced ¼ in. apart in rows spaced 4 in. apart, with 16 seeds per row and with 5 rows per flat. Vigorously growing seedlings were inoculated by the Carborundum leaf-wiping method about 6 weeks after sowing. Inoculum was freshly extracted juice of TEV-infected plants of either Holmes' Samsun tobacco (3) or of L-gene peppers (2), both of which localize tobacco-mosaic virus, thus avoiding contamination with it. The crude extracts were used undiluted or diluted ½ with distilled or with tap water. Although all inocula that were used gave good results, the author prefers extracts prepared from young vigorously growing tobacco plants 10–14 days after inoculation, since Holmes (4) has shown that the virus titre in the juice is highest at this time.

Inheritance in C. frutescens P. I. 152225.—To aid with the genetic analysis of resistance in this species, use was made of the unique sensitivity of the Tabasco pepper to infection wtih TEV. This is a dominant, usually lethal, wilt reaction in F_1 hybrids of Tabasco and any resistant form of *C. frutescens;* hence, its use simplifies the classification of the seedlings in segregating populations, especially in the backcross to P. I. Disease readings were made 9 and 21 days after inoculation. The 9-day figures were based on 4 classes of disease reaction: 1) symptomless; 2) apical mottling and veinclearing; 3) apical mottling, veinclearing, and wilting; and 4) wilting without discernible mottling. The 21-day readings were based on 5 classes: 1) symptomless; 2) slight mottling; 3) medium to severe mottling, with or without wilting; 4) wilting and defoliation, followed by limited recovery; and 5) wilting and dying. In the 9-day tabulation, only class 1 plants were considered resistant; in the 21-day tabulation, only class 1 and class 2 plants were so considered. Classes 2 and 3 in the 5-class system caused some difficulty, because some class 3 plants that were mottled but vigorous and only mildly affected should perhaps have been placed in class 2. The average percentage germination of 31 samples of the F_2 and of 9 of the backcross to P. I. was 79.2 (393 of 496) and 88.9 (128 of 144), respectively. The percentage of germinated seedlings used was 97.7 (384 of 393) and 98.4, (126 of 128), respectively.

All plants were inoculated on from 1–5 (mostly 2) leaves each with tobacco extract, and the plants were protected from the direct rays of the sun for several days.

Primarily as a check on potency of the inoculum but also for completeness, 11 plants of the Tabasco pepper, 5 of the F_1 P.I. × Tabasco, and 45 of the backcross of the F_1 to Tabasco were included. Potency of the inoculum was proved by the prompt wilting of all these plants.

The results from the critical populations, namely, the backcross to P. I. and the F_2, are presented in Table 1. The results of the 9- and 21-day tabulations were very similar, indicating that the separation of susceptible from resistant plants was sharp and that symptoms developed promptly. That this separation corresponds to the distribution of a single pair of alleles seems obvious from the backcross data. It is confirmed by the generally high chance probabilities of the 9 individual X^2 values (not shown) for tests of the 1:1 ratio, the high chance probability of the pooled value of the 9 X^2, the high chance probability of the single X^2 based on total counts (all indicating close fit of the 1:1 hypothesis), and the high chance probability of the X^2 for heterogeneity, indicating that the 9 backcross samples were samples from a single or homogeneous population.

In the F_2, there was a uniform trend of a deficiency of resistant individuals over the 31 samples. This was evident from the highly significant X^2 for a 3:1 ratio, and from the nonsignificant heterogeneity X^2 (Table 1). The homogeneity of the 31 F_2 samples considerably increases confidence in the hypothesis that the deficiency of resistant individuals is an inherent property of this particular F_2 population. A probable explanation of this deficiency is presented in the discussion.

Inheritance in C. annuum S. C. 46262.—Two essentially identical tests, differing only in the source of inoculum, were conducted with S. C. In the first, seeds were sown on October 14, and the plants were inoculated on November 15 with undiluted juice from tobacco plants that had been infected 10 days. The percentage of seedlings obtained from the 11 backcross samples was 87.5 (154 of 176), and the percentage of seedlings sampled was 98.7 (152 of 154). The corresponding figures for the F_2, including 45 albino seedlings that died, were 85.0 (272 of 320) and 96.8 (211 of 218), respectively.

Only 2 classes of disease reaction, namely, resistant (symptomless) and susceptible (mottled) were recognized in this test. A few small, unclassifiable, and broken or blind seedlings were omitted. With few exceptions, a precise classification was possible. Plants were classified 16 and 22 days after inoculation. Both dates gave similar results; hence, the X^2 were calculated only for the 22-day figures. Twelve Pimiento,

TABLE 1.—*Chi-square values and probabilities for 9 backcross (1:1 hypothesis) and 31 F_2 (3:1 hypothesis) samples from crosses of Capsicum frutescens (P. I. 152225) classified for resistance to tobacco-etch virus 9 and 21 days after inoculation*

Population	Days after inoculation	Observed ratio[a] s/r	X^2 tested[b]	D.F.[c]	X^2	Percentage probability[d]
F_1 (P.I. 152225 × Tabasco) × P.I. 152225	9	63/62	Pooled	9	8.257	58.8
			Total	1	0.008	93.3
			Heterogeneity	8	8.249	41.7
	21	68/58	Pooled	9	8.421	49.3
			Total	1	0.794	39.0
			Heterogeneity	8	7.627	47.4
F_2 (P.I. 152225 × Tabasco)	9	310/78	Pooled	31	21.645	86.2
			Total	1	4.962	0.03*
			Heterogeneity	30	16.683	97.5
	21	313/71	Pooled	31	24.835	73.1
			Total	1	8.681	0.01*
			Heterogeneity	30	16.154	98.1
			Heterogeneity[e]	30	20.472	90.3

[a] Ratios are number of susceptible (s) / number of resitant (r) plants.
[b] Three Chi-square (X^2) were tested: 1) the pooled or summed X^2 of the individual sample X^2, 2) the X^2 of the total ratio, and 3) the heterogeneity X^2.
[c] Degrees of freedom.
[d] Percentage probability of a X^2 as large as that quoted but due to chance alone. An asterisk indicates that X^2 deviates significantly from expectation.
[e] The correct heterogeneity X^2 as obtained by calculation from the actual ratio 313:71 rather than from the non-fitting hypothetical 3:1 ratio.

26 F_1 S. C. × Pimiento, and 31 backcross plants (to Pimiento) served as the susceptible checks; they promptly became mottled, whereas 18 progeny of S. C. P_2 × self remained symptomless.

In the second test, the seedlings, 38 days from seed, were inoculated on January 18. Inoculum was extract, diluted ½ with tap water, from L-genotype pepper plants (infected 64 days) of the backcross to the resistant parent. The percentages of seedlings that were obtained from the F_2 and backcross seeds were 72.7 (291 of 400) and 89.6 (43 of 48), respectively, not including 5 seedlings from 16 poor seeds of the latter. The percentages of seedlings that were sampled from each were 98.3 (286 of 391) and 97.9 (47 of 48), respectively. Nine Tabasco pepper seedlings, 5 seedlings of *Datura ferox*, and 5 of *D. stramonium* were inoculated as checks near the end of the test. The 9 Tabasco seedlings were wilted 7 days after inoculation, and the 5 of *D. ferox* showed distinct veinclearing in the shoot on the eighth day. The 5 plants of *D. stramonium* had developed a mottle by the 17th day. All seedlings from the resistant parent selections remained symptomless.

The plants were scored 14 and 25 days after inoculation. Results of the tabulations of both dates were similar. The 22-day data from test 1 and 25-day data from test 2 were combined in 1 X^2 analysis shown in Table 2.

EFFECTS OF REPEATED INOCULATION.—A second inoculation of resistant F_2 segregates, made about 21 days after the first, produced additional infected plants with symptoms that varied in severity. Some plants remained symptomless, whereas others showed various degrees of mild mottling (Fig. 1). A few plants developed a severe systemic disease with some necrosis of leaf tips and brown spots at the leaf bases. Three weeks after the second inoculation, the F_2 ratio of susceptible to resistant plants had shifted from 164:47 to 174:33. By contrast, the backcross (to the resistant parent) ratio (88:63) remained unchanged after similar treatment, indicating the presence of modifier genes in S. C. The selfed progeny of the resistant parent remained symptomless.

RESULTS OF SUBINOCULATION TESTS WITH RESISTANT PLANTS.—Subinoculations to Tabasco from 18 resist-

TABLE 2.—*Chi-square values and probabilities for 15 backcross (1:1 hypothesis) and 44 F_2 (3:1 hypothesis) samples from crosses of Capsicum annuum var. Cayenne (South Carolina 46252) classified for resistance to tobacco-etch virus 22 or 25 days after inoculation*

Population	Observed ratio[a] s/r	X^2 tested[b]	D.F.[c]	X^2	Percentage probability[d]
S.C. 46252 P_2[e] × (F_1 Pimiento × S.C. 46252 P_2)	113/86	Pooled	15	16.505	35.5
		Total	1	3.663	5.8
		Heterogeneity	14	12.842	53.9
F_2) Pimiento × S.C. 46252 P_2[e]	380/117	Pooled	44	42.775	93.8
		Total	1	0.564	46.5
		Heterogeneity	43	42.211	97.5

[a] Ratios are the number of susceptible (s) / number of resistant (r) plants.
[b] Three Chi-square (X^2) were tested: 1) the pooled or summed X^2 of the individual sample X^2, 2) the X^2 of the total ratio, and 3) the heterogeneity X^2.
[c] Degrees of freedom.
[d] Percentage probability of a X^2 as large as that quoted but due to chance alone.
[e] Highly TEV-resistant single-plant selections used in crosses.

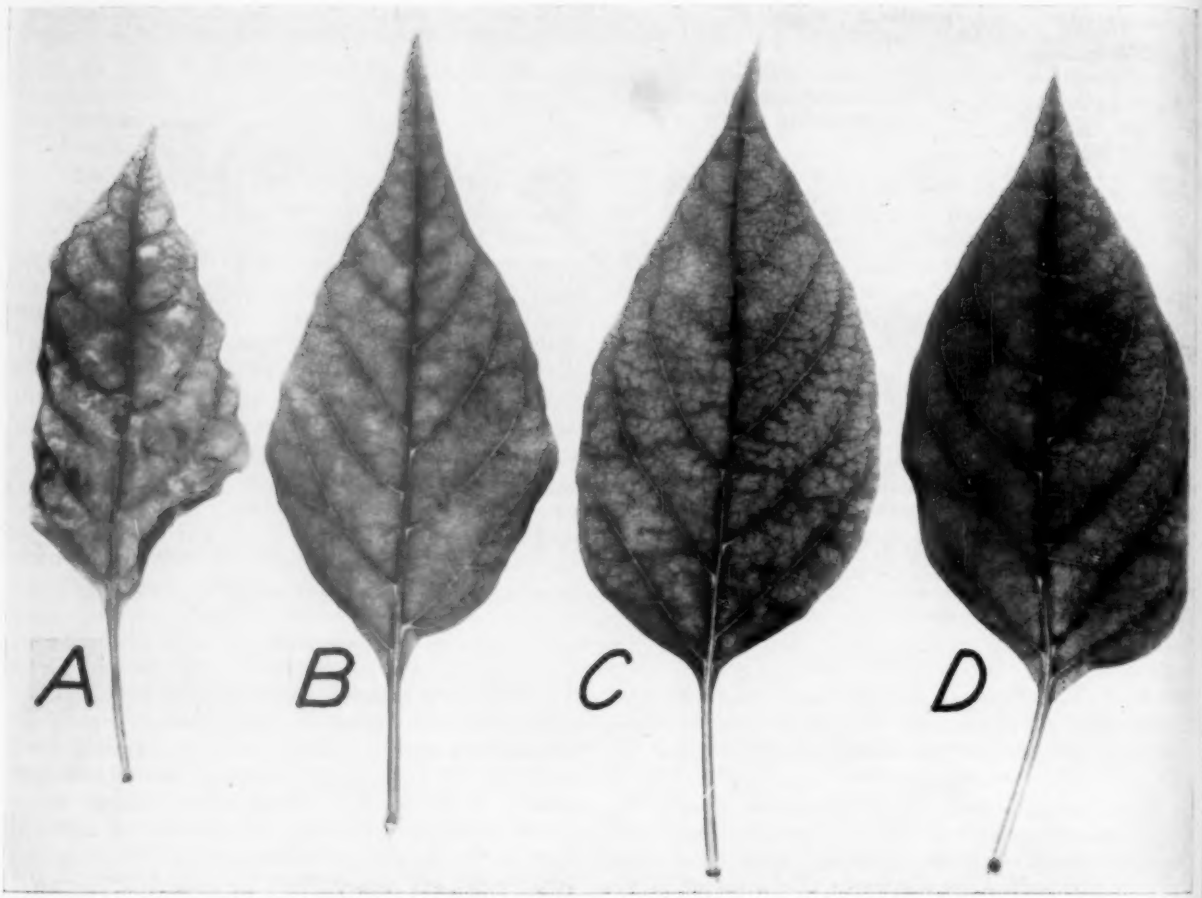

FIG. 1. Symptom expression in selections of F_2 (Pimiento × Cayenne S. C. 46252) inoculated once with tobacco-etch virus on January 18 and photographed 80 days later. A) Leaf from a susceptible plant showing mottled, wavy-margined leaves. B, C, D) Leaves from resistant plants. Note chlorosis in B, veinbanding in C, and indistinct symptoms in D. Subtests to the Tabasco pepper of the plant in B were negative on the 58th day after inoculation but positive on the 73rd day. Tests of plants as in C were always positive, whereas those of plants as in D could be either positive or negative.

ant F_2 plants that had been inoculated once or twice and sampled variously after 45, 58, and 73 days showed 14 positive and 4 negative. These 18 F_2 plants had been superficially symptomless at the time of sampling (Fig. 1), when most of them showed a faint veinbanding mottle visible by transmitted light and others were symptomless or showed uncertain symptoms. Whereas virus carriers usually could be correctly diagnosed visually, this method was not always a reliable one. One of the 18 F_2 plants mentioned had tested negative when sampled 58 days after inoculation, but it gave a positive test when sampled again on the 73rd day, indicating that virus multiplication in it was very slow (Fig. 1).

To determine whether symptomless, apparently immune, selections of S. C. would breed true, the once- or twice-inoculated selfed progeny from 2 such selections and a mixture of selfed and intercrossed progeny from a third were sampled on Tabasco. TEV carriers were detected in each of the 3 essentially symptomless samples, 18 of 28 tested plants proving positive; however, some plants of S. C. and a few also of the F_2 still seemed immune.

In a final test to determine whether some of these highly resistant plants were actually immune, 3 selections of S. C. and 4 of F_2 were again inoculated heavily on from 26–47 leaves per plant with a 50:50 tobacco extract—tapwater preparation from a 38-day source. Two subtests to Tabasco were made after 35 and after 67 days to determine whether virus had multiplied and spread to the shoot tips. Both subtests of the 7 plants, to 5 Tabasco seedlings each, were negative.

DISCUSSION.—*The inheritance of resistance to TEV in C. annuum S. C. 46252.*—The resistance of S. C., recessive to susceptibility, proved heritable by its recovery in F_2 and in the backcross to S. C. The ratios in the F_2 and in the backcross gave a satisfactory fit with expectation based on the 3:1 and 1:1 hypotheses, respectively. Accordingly, a major, single

recessive gene pair designated et^s/et^s, the superscript denoting origin from *C. annuum*, is postulated to determine resistance in S. C. The dominant allele, designated et^{s+}, is postulated to determine susceptibility. The gene et^s is interpreted to effect a slow rate of TEV multiplication, thus differentiating the resistant from the susceptible class in the 21-day classification. This would explain the increased number of carriers with time. In addition, 1 or more modifying genes are postulated to increase the resistance level to that found in the most resistant plants, i.e. in those that appeared to be immune. The author considers the existence of absolute immunity in S. C. doubtful and difficult to prove. Its attainment through further selection for minor genes for resistance, however, remains a possibility, since the original large genetic variability of response to TEV infection in S. C. (1) had been eliminated by previous selection.

The aberrant ratio in C. frutescens F_2 (P. I. 152225 × Tabasco).—The deficiency of resistant plants in the F_2 (P. I. × Tabasco) (Table 1) is probably due to the subspecific nature of this cross. That P. I. is closely related to *C. frutescens* var. Tabasco is apparent from its easy reciprocal crossability with this species and the fertility of the hybrids. The presence in P. I. of 2 flowers per axil, an important taxonomic character of *C. frutescens* (6), strengthens this evidence. The conclusion that this relationship is at the subspecies level rather than at the varietal level is evident, however, from the reduced pollen fertility of the F_1, which was 28 per cent compared to a mean of 90 per cent in the parents. The seed fertility was correspondingly reduced, the F_1, Tabasco, and P. I. having 6.3, 21.2, and 28.1 seeds per fruit, respectively, when open pollinated in the greenhouse. In a preliminary germination test, a proportion of F_2 seedlings emerged only partially from the seed coat. Many of these, extricated manually, developed abnormally, and those that survived grew slowly into highly abnormal dwarf plants with thick, distorted, and malformed leaves. Such seedlings that normally would fail to germinate are postulated to include the missing TEV-resistant plants. It is probable that a higher frequency of nonviable zygotes is produced in F_2 than in either of the backcrosses. During meiosis in the F_1 hybrid P. I. × Tabasco, pairing and crossing over between homologous chromosomes having small structural rearrangements would likely produce gametes with chromosomes having duplications, deficiencies, or both, as diagrammed by Stebbins (7, p. 224-225). Whereas the union of such gametes probably would often result in nonviable F_2 zygotes, the imbalance of such gametes probably would be "covered" frequently by the normal ones in the backcross, thus producing viable zygotes. Stebbins has termed the cause of this situation, which is common in subspecific crosses, "cryptic structural hybridity," since it is not revealed by the pairing relationship in meiosis.

Highly abnormal seedlings similar to those obtained in the F_2 (P. I. × Tabasco) also resulted from the interspecific triple cross F_2 (*C. frutescens* var. P. I. × Tabasco) × *C. annuum* var. Pimiento. Only 5 seedlings, all of the abnormal type, were obtained from about 120 seeds of this cross.

Inheritance of resistance to TEV in C. frutescens P. I. 152225.—Inheritance of resistance in P. I. was similar to that in S. C. A single recessive gene pair et^r/et^r, the superscript denoting origin from *C. frutescens* L., is postulated to determine a slow rate of TEV multiplication, whereas the dominant allele, designated et^{r+}, is postulated to determine rapid multiplication. No assays were made on the Tabasco pepper of individual inoculated plants of P. I. to ascertain if differential resistance levels, determined by modifier genes, also exist among plants of this species as was the case in S. C. Hence it is not known if near-immunity (actual immunity?) might not also be found in P. I. as it was in S. C.; however, the resistance level in P. I. appears to be lower than that of the most resistant plants of S. C. Moreover, the interspecific sterility barrier makes the use of P. I. in the breeding of resistant varieties of *C. annuum* difficult.

DEPARTMENT OF HORTICULTURE
ALABAMA AGRICULTURAL EXPERIMENT STATION
AUBURN, ALABAMA

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PRATYLENCHUS VULNUS, PRIMARY CAUSE OF THE ROOT-LESION DISEASE OF WALNUTS [1]

B. F. Lownsbery [2]

SUMMARY

The root-lesion disease of walnuts was produced experimentally by addition of a suspension of *Pratylenchus vulnus* Allen & Jens. to seedlings of *Juglans hindsii* Jepson. When a portion of the suspension was passed 7 times through a screen with 43-μ openings before addition to the walnut seedlings, no disease resulted. The repeated screening procedure removed nearly all of the root-lesion nematodes, but did not remove bacteria or fungi. Therefore it is concluded that *P. vulnus* is the primary pathogen.

The disease was also reproduced by growing seedlings of *J. hindsii* in cans of soil from an affected orchard. Air-drying this same soil eliminated *P. vulnus* and the disease.

Leaves from the diseased seedlings in both tests were lower in potassium content than leaves from healthy seedlings.

It is estimated (2) that approximately 50,000 acres of Persian walnuts, including those in all important walnut-growing areas in California, are infested with *Pratylenchus vulnus* Allen & Jens. (3). This root-lesion nematode species also attacks a large number of other woody and herbaceous plants (6, 7, 8) and has been found in widely separated locations across the United States (12). It is associated with a walnut disease characterized by stunting, dieback, and chlorosis in the top of the plant; by yield reduction; and by root symptoms consisting of black lesions, longitudinal cracking, or blackening and death of entire roots (1, 11). *P. vulnus* can be recovered from such roots (1). Root-lesion formation is a characteristic effect of activity of members of the genus *Pratylenchus;* e.g. *P. minyus*, freed from other organisms, has been observed to produce lesions on tobacco roots grown in otherwise sterile culture (9). Walnuts grown on Paradox hybrid rootstocks, which resist lesion formation, are more vigorous than walnuts grown on California black walnut, *Juglans hindsii*, which is susceptible to lesion formation (11). *Pteryocarya stenoptera* DC., wingnut, grown in affected orchards resists lesion formation and disease (11).

This evidence implicates *P. vulnus* as the probable primary pathogen. It can be argued, however, that 1) association is only a first step in proof of pathogenicity; that 2) the better performance of Paradox hybrids and *Pteryocarya stenoptera* may be a direct result of their vigor rather than of their resistance to lesion formation; and that 3) the observed correlation between tree vigor and population level of *P. vulnus* in the field is not always inverse and high.

For these reasons, controlled experimental tests of the relation of *P. vulnus* to walnut growth were deemed desirable. Two such tests have been made. In the first, studies were made of the effects of addition of *P. vulnus* to a soil previously freed from pathogens with Dowfume MC-2.[3] In the second test, growth of walnut seedlings in orchard soil infested with the root-lesion nematode was compared with growth in the same soil freed of *P. vulnus* by air drying. Leaves from both tests were analyzed chemically to learn more about the symptomatology of the disease, and to gain introductory clues to possible mechanisms of pathogenesis.

EFFECTS OF ADDITION OF P. VULNUS.—*Materials and methods.*—Twelve seedlings of *J. hindsii* grown from stratified, hulled, and disinfested (mercuric chloride 1:1000 for 5 minutes) nuts were planted in 12 ½-gal. cans of thoroughly mixed sandy loam that had been treated with Dowfume MC-2. The planted seedlings, which had been selected for uniformity and whose tops were approximately 8 cm tall, were divided into 3 lots. A suspension containing 47,950 ± 1,222 of the root-lesion nematodes was pipetted around the base of each seedling in lot 1. The suspension was obtained by incubating (15) roots of Golden Wax bush beans (*Phaseolus vulgaris* L.) grown in soil infested with *P. vulnus*. This nematode was the only plant parasitic species in the suspension. An equal volume of the same suspension was passed 7 times through a screen with 43-μ openings and then pipetted around the base of seedlings in lot 2. Lot 3 was not treated. The 3 lots were then grown in a lathhouse in 4 randomized blocks. After 4 months, heights of seedlings were measured (Table 1), roots photographed (Fig. 1), leaves analyzed (Table 2), and root-lesion nematode populations determined (Table 1). The nematodes were extracted from the entire roots by incubation (15) at 16°C; counts were made every 2 weeks for a period of 2 months. Soil from each replicate was thoroughly mixed and *P. vulnus* was extracted from a 50-cc portion. The 50-cc sample was spread over a sheet of toilet paper held on a wire screen resting in a funnel of water.[4] Extraction was continued for 1 week. The number of root-lesion nematodes extracted from 50 cc was multiplied by the factor necessary to give the total population in the

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[2] E. F. Serr, Pomology, gave advice regarding walnut seedling culture. J. G. Brown, Pomology, made leaf analyses. Starr T. Miyagawa assisted as technician.

[3] Methyl bromide (bromomethane)—98 per cent; chloropicrin (trichloronitromethane)—2 per cent.

[4] This modification of the Baermann extraction was devised by Dr. R. C. Baines for use with the citrus nematode. The toilet paper used was Crown Zellerbach "No Waste."

TABLE 1.—*Heights of seedlings of Juglans hindsii and populations of P. vulnus in soil treated with Dowfume MC-2 4 months after addition of a suspension of P. vulnus or a portion of the same suspension screened to remove most nematodes*

Material added to soil	Number of root-lesion nematodes added	Seedling height (cm)	Number of root-lesion nematodes recovered
Suspension	47950±1222	10.9	2400±164
Screened suspension	154±57	21.6	376±235
Nothing	0±0	22.9	0±0
LSD at 5 per cent level		7.0	
LSD at 1 per cent level		10.5	

TABLE 2.—*Mineral content of composite leaf samples from seedlings of J. hindsii grown in soils containing various population levels of P. vulnus*

Treatment	Initial number of root-lesion nematodes/can	Mineral content as percentage of dry weight					
		K	Ca	Mg	Na	P	Mn
Nematode suspension	47,950	0.83	1.20	1.01	0.06	0.178	0.014
Screened nematode suspension	154	1.18	0.83	0.60	0.03	0.188	0.015
Infested soil	18,300	0.68	0.98	1.15	0.05	0.146	0.014
Infested soil air dried	0	1.15	1.14	0.93	0.04	0.153	0.011

can. This product was added to the extraction total from the corresponding root to give the total for root-lesion nematodes recovered when the test was terminated (Table 1).

Results.—Root symptoms of seedlings receiving the screened nematode suspension and those of seedlings receiving no treatment appeared identical. Some fine feeder roots were dead, but there was an abundance of healthy ones. The epidermis was yellow-brown and healthy, for the most part, and the cortex was a healthy yellow. Root systems of seedlings receiving the unscreened nematode suspension lacked feeder roots, and most of the epidermis and cortex was black (Fig. 1). Repeated passage of the nematode suspension through the screen with 43-μ openings removed most of the root-lesion nematodes (Table 1), but did not remove bacteria or fungi. Since this passage also made the suspension ineffective in causing disease, it is apparent that *P. vulnus* is the primary pathogen.

Pratylenchus vulnus is an obligate parasite feeding and reproducing only on living roots, especially the young "feeder" roots of its hosts. The walnut seedlings that received the unscreened suspension were subjected to a higher population of the parasites than they could support. Damage was excessive (Fig. 1), and the root-lesion nematode population fell below the number added (Table 1).

Results of chemical analyses of leaf samples (Table 2) are considered in a subsequent section, together with similar analyses from the second experiment.

EFFECTS OF AIR DRYING ORCHARD SOIL INFESTED WITH P. VULNUS. — *Materials and methods.* — Soil from a Davis, California, walnut orchard infested with *P. vulnus* was passed through a coarse screen and thoroughly mixed. One half of this soil was spread in a thin layer in full sunlight and left for 4 days. The other half was stored, moist, at 16°C for this period. Five 3-gal. cans of soil from each of the 2 treatments were placed as 5 pairs in a lathhouse and planted August 16, 1954, with seedlings of *Juglans hindsii* selected for uniformity from a lot that had been stratified and grown in soil and sand treated with Dowfume MC-2. They were approximately 22 cm tall when transplanted.

Results.—At the end of the 1954 growing season, there was no difference in the growth of seedlings in air-dried soil and growth in untreated soil. By May 12, 1955, terminal growth in air-dried soil was sig-

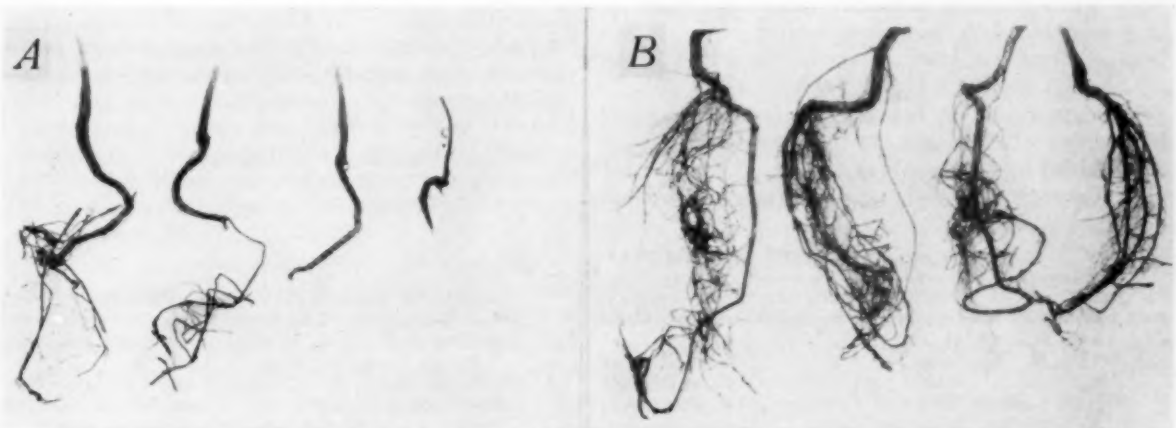

FIG. 1. Roots of seedlings of *J. hindsii* grown in soil to which was added A) an untreated portion of a suspension containing 47,950 nematodes (*P. vulnus*) and B) an equivalent portion of the same suspension screened to remove most of the nematodes.

TABLE 3.—*Terminal growth of seedlings of J. hindsii and populations of P. vulnus in air-dried and untreated walnut orchard soil before and after growth of the seedlings*

Treatment	*P. vulnus* (number/can)		Terminal growth (cm) (1955)
	Initial	Final	
Air dried	0	0.8±0.5	22.4
Untreated	18,300±1500	71,899±14,480	13.2
LSD at 5 per cent level			7.6
LSD at 1 per cent level			10.9

nificantly greater than in untreated soil. The difference was still evident when the experiment was terminated July 25 (Table 3). The cork of roots grown in air-dried soil was yellow-brown and intact, and there was an abundance of healthy feeder roots. Scraping the cork uncovered only yellow healthy phloem. Root systems from untreated soil had few feeder roots, and those that were present showed considerable necrosis. Scraping of the cork of the primary roots revealed black lesions (Fig. 2) that usually extended only into the phloem but sometimes extended into the xylem. The seedlings in this test were larger than those used in the first test involving addition of *P. vulnus*, and the nematode population to which they were subjected was smaller. Root damage in this experiment was less, therefore, and there was sufficient food to allow a 4-fold increase in the nematode population (Table 3).

The orchard soil used in this test also contained small numbers of the nematodes *Criconemoides xenoplax* Raski and *Xiphinema americanum* Cobb. These remained at a low level, and are not believed to have been an important confounding factor.

Potassium content of leaves from the untreated nematode-infested soil was less than that of leaves from air-dried nematode-free soil (Table 2). Potassium content was also lower in leaves of those seedlings that were exposed to a large number of root-lesion nematodes in the first test (Table 2). It appears likely that the nematode causes this decrease in potassium. Low potassium content of leaves has been observed for other nematode diseases (10, 13, 14), but an exception to this has been noted (5).

DISCUSSION.—In the first test, disease was produced by addition of *P. vulnus*. In the second test, disease was avoided by killing this nematode. The ineffectiveness of components of the nematode suspension other than the nematodes themselves in producing the root-lesion disease of walnuts leaves *P. vulnus* as the only likely primary pathogen. Some of the symptoms of the root-lesion disease of walnuts may be the result of activity of secondary pathogens, but blocking the nematode appears to be the most probable key to therapy.

FIG. 2. Lesion on primary root of a seedling of *J. hindsii* grown in untreated orchard soil infested with *P. vulnus*.

The fluctuation in root-lesion nematode population levels in these experiments has been related to food supply. Availability of food also explains why nematode populations can seldom be correlated with apparent damage in the orchard. Little food will be available in a heavily damaged root system, whereas an abundance of food is available when damage is not so far advanced.

Potassium deficiency in walnuts and the root-lesion disease of walnuts both show the dieback symptom, but the leaf scorch reported for simple potassium deficiency (4, p. 481) has not been observed for the root-lesion disease. It is possible that a portion of the symptomatology of this disease results from potassium deficiency brought about by the nematode's use of the element, the nematode's effect on the root's potassium-absorbing capacity, or both.

DEPARTMENT OF PLANT NEMATOLOGY
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA

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ASSOCIATION OF RESISTANCE TO SEVERAL SEEDLING, ROOT, STALK, AND EAR DISEASES IN CORN[1]

A. L. Hooker[2]

SUMMARY

The disease reactions of 25 dent corn inbred lines to artificial inoculations that resulted in basal stalk rot incited by *Diplodia zeae*, in basal stalk rot incited by *Gibberella zeae*, and in basal stalk rot incited by both *D. zeae* and *G. zeae* (simultaneous inoculations) were measured in 1953 and 1954. Also measured were the reactions of the inbred lines to natural infections that resulted in basal stalk rot, in root necrosis, in ear rot incited by *D. zeae*, and in ear rot incited by *G. zeae*. Seedling reactions to artificial inoculations with *Pythium debaryanum*, with *P. graminicolum*, with *D. zeae*, and with *G. zeae* were determined in the greenhouse.

Differences among corn strains in all evaluations were statistically significant. Highly significant correlations were measured among the stalk rots, including those with root necrosis, and among the 4 seedling blights. Correlations involving stalk rot in the internode below the ear with the remaining stalk rots and with root necrosis were low in 1953. The 2 ear rots incited by *D. zeae* and by *G. zeae* were not correlated with each other or with other disease reactions. Correlations between seedling blights and stalk rots or root necrosis were negative but statistically nonsignificant.

Seedling blight and root, stalk, and ear rots of corn (*Zea mays* L.) are widespread and often cause appreciable losses. *Diplodia zeae* (Schw.) Lév., *Gibberella zeae* (Schw.) Petch, and several species of *Pythium* are generally regarded as being the most prevalent and destructive pathogens in this disease group, although other fungous and bacterial species have been reported as pathogens of these plant parts. *Pythium debaryanum* Hesse and *P. graminicolum* Subr. are among the *Pythium* species that generally attack only seedlings and roots.

With the exception of chemical seed treatment for seedling blights, breeding for resistance is the most feasible control for this group of diseases. Modern corn breeding methods involve the isolation of inbred lines or of their equivalents and the recombination of these lines into hybrids. Hybrids are resistant usually in proportion to the number of resistant inbred lines used in their synthesis. The breeding objective, therefore, is the isolation of suitably resistant inbred lines. It seemed desirable to determine if the reaction of one strain of corn at one stage of development to one pathogen might be associated with its reaction to other pathogens at the same stage, or whether resistance to one pathogen in one plant part might be correlated with resistance to the same pathogen in other plant parts. It was hoped that such information would lead to a simplification or clarification of the breeding objectives in regard to this group of diseases.

Ullstrup (19) has recently reviewed the literature and cited several reports of resistance to 1 or more diseases in this group. Smith, Hoppe, and Holbert (18) reported a successful inoculation technique for stalk rot incited by *D. zeae* and reported a highly significant association between spread of *D. zeae* in inoculated plants and percentage of infected plants or percentage of broken plants in the noninoculated

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[2] Present address, Department of Plant Pathology, University of Wisconsin, Madison 6, Wisconsin.

plots. In addition, various investigators have reported the following relationships or associations in disease response: corn borer feeding and stalk rot (2), spread of *D. zeae* and *G. zeae* in inoculated stalks (16; P. E. Hoppe, unpublished data), spread of *D. zeae* in inoculated stalks and number of dead plants resulting from natural causes (17), seedling blight and stalk rot in relation to resistance to *D. zeae* (15), and resistance in the seedling stage to several *Pythium* species (8). Suggestions or evidence has been presented for the lack of association in the following: seed infection with *D. zeae* and ear rot (3) or stalk rot (3, 4, 12), seed infection with *G. zeae* and root rot (1), and seedling blight in the greenhouse and stalk rot in the field, both incited by *D. zeae* (17).

MATERIALS AND METHODS.—Twenty-five dent corn inbred lines, representing a range of maturities and a diversity of origin, were selected for this study. These included 38-11, 187-2, A73, B2, B6, B10, B14, B36, C103, Hy-2, Ia153, L317, M14, N6, Oh07, Oh41, Oh43, Os420, W22, and WF9, all of which had been selfed for many generations, and the lines 82, 225, 502, 698, and 829, which were derived by the monoploid method. All were highly homozygous, and many are widely used in commercial hybrid production.

Each of the inbred lines was evaluated for its reactions to artificial inoculations that resulted in Diplodia basal stalk rot incited by *D. zeae*; in Gibberella basal stalk rot incited by *G. zeae*; in "Diplodia and Gibberella" basal stalk rot incited by both *D. zeae* and *G. zeae*; in Diplodia stalk rot in the internode below the ear, incited by *D. zeae*; and in 4 seedling blights incited by *D. zeae*, by *G. zeae*, by *P. debaryanum*, and by *P. graminicolum*. Evaluations were made also with respect to natural infections that resulted in basal stalk rot, root necrosis, and 2 ear rots (incited by *D. zeae* and by *G. zeae*). Disease ratings for the stalk rots, ear rots, and root necrosis were obtained in the field in 1953 and again in 1954. A split, split plot experimental design with 4 replications was employed. Ten individual plants in each entry were scored for the stalk rots; 5 plants for root necrosis, and 75 plants for the ear rots. Ratings for the 4 seedling blights were obtained in replicated experiments in the greenhouse.

The inoculations for Diplodia stalk rot were made by injecting a water suspension of pycnospores into the third internode above the ground for the basal stalk rot or in the internode below the ear. The latter inoculation was made to simulate stalk rot infection such as may follow corn borer (*Pyrausta nubilalis* (Hbn.)) feeding. Inoculations for Gibberella stalk rot were made by drilling a small hole into the third internode above the ground, inserting a mycelial mass of the fungus growing on a sterilized barley kernel, and plugging the hole with cotton. The "Diplodia and Gibberella stalk rot" evaluation consisted of the simultaneous inoculation of the same internode with the 2 pathogens. All inoculations for stalk rot were made within a 3-day period. Pollination had occurred from 1 to 2 weeks earlier, depending upon the inbred line.

Notes on the severity of stalk rots were taken 4 weeks after inoculation. Ten plants in each entry were cut longitudinally and scored individually for resistance according to the following scheme: 1) 0–25 per cent, 2) 26–50 per cent; 3) 51–75 per cent, and 4) 76–100 per cent of the inoculated internode infected with the fungus; 5) infection extending into adjacent internodes; and 6) plant killed. These 10 plant readings were averaged to obtain a score for each entry. Ratings for stalk rot due to natural infection were obtained by a similar but slightly modified scheme.

Root necrosis ratings were obtained 5–6 weeks after pollination. The crown and adjacent roots of 5 plants in each entry were observed and individually scored for root necrosis according to the following scheme: 1) 0–25 per cent, 2) 26–50 per cent, 3) 51–75 per cent, 4) 76–100 per cent of the root surface showing lesions; 5) root system dead and fragmented; and 6) root system and plant dead. These 5 plant readings were averaged for each entry.

Diplodia and Gibberella ear rot ratings consisted of the average percentage of ears infected with each fungus. These percentages were obtained from the macroscopic classification of 75 open-pollinated ears of each inbred line in each replicate.

Seedling blight ratings were obtained under controlled environmental conditions in the greenhouse. In all cases, relatively fungus-free seed was employed, and ratings were obtained on 4 replicates of 25 plants each. Inoculations with *P. debaryanum* were made by planting the seed between thin layers of crushed fungous mycelium mixed with steamed quartz sand over steamed soil in clay pots (8). These were watered to 60 per cent of water-holding capacity and were incubated at 10°C for 8 days before they were placed on the greenhouse bench for seedling development. Inoculations with *P. graminicolum* were made in a similar manner, but the pots were placed directly on the greenhouse bench. Gibberella inoculations were made by planting the seed on a very thin layer of crushed fungous mycelium mixed with steamed quartz sand, all in steamed soil and clay pots. Pots were placed in a greenhouse maintained at 16°C and the soil kept at about 60 per cent of water-holding capacity. In the inoculations for Diplodia seedling blight, seed was dipped for 10 seconds in a water suspension of pycnospores and then planted in freshly steamed soil. The pots containing the inoculated seed were placed in a 24°C greenhouse and the soil maintained at about 40 per cent of water-holding capacity.

Records of seedling blight severity were taken at the 3- or 4-leaf stage for all diseases. In each case, the seedlings were washed after removal from the soil and grouped into 6 classes according to the severity of infection (8). A resistance index was obtained by multiplying the number of seedlings falling in each class by the respective class value, which ranged from

1 (most diseased) to 6 (no observable infection), and adding the products together.

RESULTS.—*Varietal reaction.*—Differences beyond the 5 per cent level of statistical significance existed among the 25 corn strains in their reaction in each of the 12 disease evaluations (Table 1). Inbreds B2, B6, B14, C103, W22, and 187-2 were among the most resistant lines to the stalk rots and root necrosis whereas Ia153, L317, Os420, and 698 were most susceptible. All lines were relatively susceptible to Diplodia spread in the internode below the ear. The most seedling blight resistance was expressed by inbreds A73, 82, 225, 829, L317, and N6 and the least by inbreds B2, B6, B10, C103, Oh07, and WF9. Inbred lines A73,

TABLE 1.—*Average disease evaluations*[a] *for 25 dent corn strains in 1953 and 1954*

Corn strain	Year	Stalk rot and root necrosis indices of indicated diseases (6 = susceptible, 0 = resistant)						Percentage ear rot		Seedling blight indices of indicated diseases (0—susceptible, 100 = resistant)			
		1	2	3	4	5	6	7	8	9	10	11	12
A73	1953	4.6	3.8	5.0	1.9	3.9	5.4	0.0	0.0				
	1954	3.7	4.1	4.3	2.4	3.8	3.2	0.4	10.7	75	42	41	64
B2	1953	1.0	1.1	1.8	0.0	2.5	1.5	0.0	2.4				
	1954	1.2	1.1	1.2	0.2	2.7	1.0	0.7	25.9	54	22	22	20
B6	1953	1.2	2.3	3.0	0.0	3.7	2.4	1.7	0.0				
	1954	1.4	2.0	2.4	0.0	3.7	1.6	0.4	2.4	42	17	21	17
B10	1953	1.3	1.9	2.7	0.0	4.2	1.6	0.0	1.1				
	1954	1.1	1.8	2.1	0.0	3.8	1.6	0.4	15.6	25	19	20	17
B14	1953	1.3	1.8	2.0	0.0	3.6	1.0	4.9	0.0				
	1954	1.0	1.0	1.4	0.0	3.6	1.2	0.4	7.8	72	30	33	33
B36	1953	2.5	3.1	3.4	0.0	4.7	1.0	0.0	0.5				
	1954	2.8	2.6	3.0	0.0	3.9	1.4	1.2	14.9	50	33	29	17
C103	1953	1.3	2.0	3.5	0.2	3.8	2.6	2.2	1.8				
	1954	1.2	1.5	1.9	0.0	3.0	2.4	2.6	22.6	52	18	24	20
Hy-2	1953	1.9	2.4	4.1	0.0	4.2	4.0	0.7	0.7				
	1954	3.4	2.4	3.8	0.0	4.0	2.8	0.0	15.8	63	34	38	30
Ia153	1953	5.5	4.8	5.4	4.1	4.0	6.0	0.0	0.0				
	1954	6.0	6.0	6.0	6.0	5.0	5.7	0.0	9.7	66	27	31	30
L317	1953	3.0	3.3	4.9	0.7	5.6	3.3	0.6	0.0				
	1954	3.9	3.6	4.0	0.1	4.0	3.0	0.4	9.5	73	38	41	55
M14	1953	2.2	3.2	4.7	0.2	4.7	4.4	0.0	0.0				
	1954	2.2	2.0	2.7	0.1	3.7	2.2	0.3	7.4	71	45	38	42
N6	1953	1.4	1.9	3.0	0.1	3.0	1.8	1.4	33.8				
	1954	3.6	2.5	3.2	0.8	3.8	3.2	1.5	15.2	72	43	44	59
Oh07	1953	2.1	2.3	4.1	0.0	4.4	2.6	0.3	1.6				
	1954	2.6	2.2	2.7	0.6	3.9	2.4	0.4	6.7	58	36	23	18
Oh41	1953	3.9	4.2	4.9	2.1	4.9	3.5	3.6	0.8				
	1954	3.0	2.8	3.5	0.0	4.0	2.4	0.0	8.1	71	38	46	51
Oh43	1953	2.3	2.6	4.4	0.1	4.1	2.3	0.3	0.0				
	1954	3.1	2.9	3.0	0.1	3.7	2.7	0.4	13.4	66	36	39	56
Os420	1953	4.6	3.9	4.4	2.8	4.0	5.6	0.0	4.1				
	1954	4.5	4.2	4.4	0.6	4.0	4.0	0.4	17.2	60	22	34	49
W22	1953	2.4	2.3	4.8	0.1	4.6	3.6	0.0	3.4				
	1954	1.5	1.6	2.4	0.0	3.3	2.7	0.8	16.3	77	44	42	37
WF9	1953	2.3	3.2	4.2	0.0	4.0	2.9	0.7	0.3				
	1954	2.0	1.8	2.6	0.0	3.7	2.4	0.0	20.3	59	27	22	18
38-11	1953	2.1	2.8	3.1	0.0	4.3	1.4	0.3	2.1				
	1954	3.1	2.0	3.1	0.0	4.0	2.1	0.8	26.2	61	37	37	47
187-2	1953	3.2	3.0	4.4	1.2	4.2	3.6	0.3	2.3				
	1954	1.8	1.7	2.3	0.6	3.7	2.4	0.0	22.4	59	30	26	20
82	1953	4.6	4.4	5.1	3.2	4.6	6.0	0.0	0.0				
	1954	3.7	3.0	3.7	0.8	3.9	3.9	0.0	5.9	77	42	47	72
225	1953	3.2	3.0	4.0	0.6	4.3	4.0	1.4	0.0				
	1954	3.6	3.7	4.4	0.8	3.8	3.4	2.7	10.4	70	49	42	68
502	1953	1.9	2.0	2.6	0.0	4.6	1.7	0.5	0.0				
	1954	2.0	2.0	2.0	0.0	4.0	1.9	1.6	2.5	65	30	36	57
698	1953	3.8	3.9	4.0	1.3	4.1	4.7	0.3	0.3				
	1954	4.5	4.3	4.6	2.4	4.0	4.0	2.1	10.5	54	29	27	50
829	1953	2.6	3.1	3.8	0.0	4.4	2.2	0.0	0.0				
	1954	3.0	3.0	3.6	0.2	3.9	3.6	0.0	5.7	70	45	40	77
LSD (0.05)	1953	0.8	0.7	0.6	0.9	0.6	1.1	1.6	4.0				
	1954	0.7	0.6	0.6	0.6	0.4	0.7	1.6	12.1	14.5	9.2	10.4	13.3

[a] Each value is an average of 4 replications. Diseases (identified by numbers) and incitants are as follows: 1) basal stalk rot, *Diplodia zeae;* 2) basal stalk rot, *Gibberella zeae;* 3) basal stalk rot, *D. zeae* and *G. zeae;* 4) basal stalk rot, natural infection; 5) stalk rot in internode below ear, *D. zeae;* 6) root necrosis, natural infection; 7) ear rot, *D. zeae;* 8) ear rot, *G. zeae;* 9) seedling blight, *D. zeae;* 10) seedling blight, *G. zeae;* 11) seedling blight, *Pythium graminicolum;* 12) seedling blight, *P. debaryanum.*

TABLE 2.—*Interannual correlation coefficients (r) measuring the similarity of reaction of 25 dent corn strains to 8 diseases in 1953 and in 1954*

Disease evaluation	r[a]
Basal stalk rot incited by *Diplodia zeae*	+0.79**
Basal stalk rot incited by *Gibberella zeae*	+0.78**
Basal stalk rot incited by *D. zeae* and *G. zeae*	+0.68**
Basal stalk rot resulting from natural infection	+0.72**
Stalk rot in the internode below ear incited by *D. zeae*	+0.44*
Root necrosis resulting from natural infection	+0.78**
Ear rot incited by *D. zeae*	+0.12
Ear rot incited by *G. zeae*	+0.18

[a] Double asterisks indicate significance at the 1 per cent level, single at the 5 per cent level.

Ia153, 82, 829, M14, and 187-2 and inbred lines B6, B14, 82, 502, 829, and M14 expressed the lowest percentages of ear rots incited by Diplodia and by Gibberella in the 2 years. Inbreds C103, N6, and 225 had the highest percentages of Diplodia ear rot and inbreds B2, Os420, N6, 187-2, and 38-11 showed considerable Gibberella ear rot.

Stalk rots and root necrosis.—Good agreement existed between the 1953 rankings and the 1954 rankings for the stalk rots and root necrosis, as indicated by the large, positive, and highly significant interannual correlation coefficients (Table 2). This was true even though a higher level of stalk rot spread was obtained in 1953 than in 1954.

Correlation coefficients (r values) were calculated to measure the similarity of reaction of the 25 corn strains to the stalk rots and to root necrosis in 1953 and in 1954 (Table 3). The r values involved in paired comparisons among Diplodia stalk rot, Gibberella stalk rot, "Diplodia and Gibberella" stalk rot, stalk rot resulting from natural infection, and root necrosis were all large and statistically significant. The r values involving comparisons with Diplodia stalk rot spread in the internode below the ear were all positive but somewhat smaller. This was particularly true in 1953 when conditions were more favorable for stalk rot development and all strains expressed susceptible ratings in this evaluation. In 1954, less stalk rot developed and larger differences among corn strains were evident in this evaluation.

Seedling blights.—As indicated by the correlation coefficients presented in Table 3, the reaction of these

TABLE 3.—*Correlation coefficients measuring the similarity of varietal reactions of 25 dent corn strains to 12 diseases in various paired comparisons in 1953 and in 1954*

Disease no.	Diseases and pathogens	Correlation coefficients[a] between indicated paired combinations of diseases									
		2	3	4	5	6	7	8	9	10	12[b]
		(1953 Evaluations)									
1	Basal stalk rot, *Diplodia zeae*	+0.93***	+0.78**	+0.91**	+0.33	+0.84**	−0.26		−0.40[c]		
2	Basal stalk rot, *Gibberella zeae*		+0.81**	+0.82**	+0.46**	+0.78**		−0.03		−0.26[c]	
3	Basal stalk rot, *D. zeae* and *G. zeae*			+0.64**	+0.55**	+0.80**					
4	Basal stalk rot, natural infection				+0.12	+0.82**					
5	Stalk rot in internode below ear, *D. zeae*					+0.20					
6	Root necrosis, natural infection								−0.36[c]	−0.20[c]	−0.32[c]
7	Ear rot, *D. zeae*							+0.07	−0.12[c]		
8	Ear rot, *G. zeae*									−0.16[c]	
9	Seedling blight, *D. zeae*									+0.77**	+0.67**
10	Seedling blight, *G. zeae*										+0.69**
11	Seedling blight, *Pythium graminicolum*					−0.31[c]			+0.83**	+0.80**	+0.84**
		(1954 Evaluations)									
1	Basal stalk rot, *D. zeae*	+0.94**	+0.96**	+0.69**	+0.76**	+0.87**	−0.03				
2	Basal stalk rot, *G. zeae*		+0.96**	+0.79**	+0.75**	+0.87**		−0.29			
3	Basal stalk rot, *D. zeae* and *G. zeae*			+0.71**	+0.77**	+0.89**					
4	Basal stalk rot, natural infection				+0.61**	+0.75**					
5	Stalk rot in internode below ear, *D. zeae*					+0.64**					
7	Ear rot, *D. zeae*							+0.11			

[a] Double asterisks indicate statistical significance at the 1 per cent level.
[b] Disease No. 12 was seedling blight incited by *Pythium debaryanum;* all other diseases are indicated by the corresponding numbers in column 1.
[c] Sign reversed to indicate true relationship between the 2 diseases.

corn strains to the 4 seedling blights were all highly associated. Inoculation techniques and environmental conditions were such that a high level of disease was encountered in each of the seedling blight evaluations. Much of the injury was in the form of a kernel rot or a pre-emergence blight. Check plantings of these corn strains in steamed soil resulted in only minor differences in number of healthy seedlings produced, indicating that stand reductions and diseased seedlings in the inoculated series were largely due to the pathogens used and not to seed-borne fungi or low seed viability.

Ear rots.—Although statistically significant differences existed among these corn strains in percentages of ears infected with *Diplodia* or with *Gibberella* (due to natural infection) each year, there was little association from year to year. Both interannual correlation coefficients (Table 2) were positive but low. Positive but statistically nonsignificant correlation coefficients were obtained each year between the prevalence of Diplodia and Gibberella ear rots, indicating that host reactions to these 2 diseases were not highly associated.

Diseases involving several plant parts.—The correlation coefficients between various paired combinations of seedling blights, stalk rots, root necrosis, and ear rots incited by a single pathogen were all negative (Table 3). Those involving seedling blights with stalk rots or root necrosis approached the 5 per cent level of statistical significance. Auxiliary experiments involving 120 paired comparisons of *Pythium* seedling blight (incited by *P. debaryanum*) resistance and Diplodia stalk rot with 120 established dent corn inbred lines, most of which were established without a uniform stalk rot evaluation, resulted in a similar negative association ($r = -0.348^{**}$). However, in 159 similar comparisons with early generation lines established in conjunction with a routine stalk rot evaluation and selection each generation, these 2 reactions were not adversely correlated ($r = -0.063$). When the 2 sets of data were combined (279 paired comparisons), a correlation coefficient of −0.018 was obtained.

Discussion.—The data presented suggest that 4 primary breeding objectives exist with regard to this group of diseases. These are resistance to 1) stalk rot, 2) seedling blight, 3) Diplodia ear rot, and 4) Gibberella ear rot. This would be particularly true in early generations when a large volume of segregating breeding material is to be screened. Later, more precise evaluations to each specific disease may be desirable. The high association between basal stalk rot resistance and low degrees of root necrosis, the biological significance of which is not clear, suggests that a reasonable rating for root necrosis can be inferred from a stalk rot evaluation. The virulence of the pathogen, reliability of techniques, and ease and speed with which the inoculations can be carried out indicate that a Diplodia stalk rot evaluation would be the most satisfactory for the stalk rot and root necrosis group. Data presented in this paper and those presented earlier (8) suggest that resistance in corn in the seedling stage is effective against a wide range of pathogens. The practice of evaluating for Pythium resistance at low temperatures (7, 9) by planting in infested soil followed by an artificial incubation at low temperature is well established in many corn breeding programs and would seem to be an effective measurement for resistance to the seedling blight group of diseases. Resistance to Diplodia ear rot and to Gibberella ear rot appear to be independent problems. Appropriate evaluations to each would be needed if attention is to be given to these 2 diseases.

Although an association between Diplodia resistance in the seedling and in the stalk has been suggested (15), data presented in this paper and others (17) indicate that this may not always be the case. In fact, with the group of inbreds studied, in no comparison was resistance to a given pathogen in one plant part positively associated with resistance to the same pathogen in another; however, there were individual inbred lines with good resistance to the same pathogen in several parts. Part of the low association between seedling blight resistance and stalk rot resistance in the corn strains studied may be related to their relative maturities. Frequently the later maturing corn strains will show more stalk rot resistance than those maturing earlier, as was true in this study, and seed that is well matured will resist seedling-infecting pathogens better than immature seed. In this study, some of the strains most susceptible to the seedling blights were late maturing and stalk rot resistant, whereas many of the early and stalk rot susceptible lines expressed good resistance to the seedling blights. An inspection of the detailed data and those from other tests indicate that not all early lines are necessarily stalk rot susceptible nor are all late lines necessarily seedling blight susceptible. This indicates that it is possible to incorporate resistance to both seedling blights and to stalk rots in the same line; in fact, corn strains with this combination are known. It would appear that resistance to all of these corn diseases could be incorporated in the same line. In practice this may be difficult and even unnecessary.

The association between stalk rot and root necrosis, if this proves to be a general situation, poses some interesting biological questions. Are stalk rots initiated by species of *Pythium* or other soil fungi that invade the plant by way of the root system as indicated by McKeen (13) and are such fungi soon followed by the fungi generally regarded as stalk pathogens? Are the roots invaded by soil fungi that so weaken the plant that stalk-rotting fungi are able to invade the stalk, or conversely do stalk-rotting fungi attack first and weaken the plants so that the roots are easily parasitized? Perhaps plant conditions that are favorable for stalk-rotting pathogens are also those permitting an invasion of the root system? Limited observations indicate that most plants naturally infected with stalk-rotting pathogens are invaded from

the base and that these plants nearly always have badly diseased root systems.

Loss of leaf area, which presumably results in a reduction of carbohydrate reserves in the plant, has been shown to predispose the plant to Diplodia stalk rot (5) and to Gibberella stalk rot (11). These observations and the association of resistance to these 2 pathogens in the stalk, as indicated by the data presented and those of others (16), suggest that the nature of resistance to these 2 pathogens may be quite similar. Additional support for this is provided by the observations of Johann and Dickson (10), who found that the soluble substance extracted from Diplodia-resistant cornstalks inhibited both *D. zeae* and *G. zeae* in culture. The association of resistance to several seedling blights suggests that the nature of resistance to these fungi in the seedling might also be quite similar. Histological observations have revealed a similar morphological response in corn seedling tissue when invaded by *G. zeae*, when mechanically injured or when invaded by *P. debaryanum* (6, 14). The ability of a seedling to send out new roots to replace diseased ones would seem to be little related to the specific pathogen invading the plant, providing they had similar modes of pathogenicity.

The general conclusions presented in this paper pertain to the specific corn strains and pathogens studied, and interpretations beyond them have limitations. It would appear, however, that the methods and procedures presented are significant and should be expanded to include additional corn strains and corn pathogens. It is anticipated that certain exceptions to the general conclusions inferred from these data will be found. This would be particularly true if additional pathogens with different modes of pathogenicity were investigated.

Similarities in host reaction to several facultative parasites that parasitize the host in a similar manner may be common in nature. If this be true, such information would greatly expedite breeding for disease resistance in those crops. Additional research along these lines would appear to be valuable.

Department of Botany and Plant Pathology
Iowa State College
Ames, Iowa

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VAPOR ACTION OF FUNGICIDES USED IN THE CONTROL OF WHEAT BUNT [1]

Laurence H. Purdy and C. S. Holton [2]

SUMMARY

Panogen 15 and Anticarie inhibited spore germination in *Tilletia caries* on water agar by vapor action whereas Ceresan M and Agrox were ineffective in this respect. Only Panogen 15 of this group effectively controlled bunt by vapor action against spores carried on dry wheat seed. The failure of Anticarie in this regard possibly is due to a mode of action requiring initiation of spore germination, which occurs only in the presence of moisture.

The failure of the volatile products of Ceresan M to control wheat bunt in this study suggests that its total effectiveness as a seed treatment for this purpose depends upon direct contact.

Vapor action is an important factor to consider in the development and recommendation of fungicides for disease control through seed treatment. Several workers (1, 2, 3, 4) have shown this to be especially true where the inoculum is seed borne beneath or in the protective covering of hulls or other plant parts. Likewise, vapor action becomes important where low rates of application preclude complete coverage of the seed. For example, the spores of the wheat bunt fungus (*Tilletia caries* (DC.) Tul.) are surface borne and therefore subject to fungicidal action by direct contact through seed treatment. The standard rate of application for certain fungicides to wheat seed for bunt control is ½ oz. to the bushel, an amount scarcely adequate for thorough coverage of each kernel; hence, the importance of vapor action to the effectiveness of the fungicides applied at this low rate.

The purpose of this study was to determine the relative effectiveness against wheat bunt of the volatile products of several fungicides used as wheat seed treatments in the Pacific Northwest.

MATERIALS AND METHODS.—The compounds used in this study were Ceresan M (7.7 per cent *N*-(ethylmercuri-*p*-toluenesulfonanilide), Panogen 15 (2.2 per cent cyano(methylmercuri)guanidine), Agrox (6.7 per cent phenylmercuriurea), Anticarie (40 per cent hexachlorobenzene), and CP grade hexachlorobenzene. Ceresan M and Panogen 15 are standard mercury treatments for wheat seed at ½ and ¾ oz. per bushel, respectively. At these low rates of application, their total effectiveness against wheat bunt is said to depend in part on the fungicidal properties of their volatile products. The volatile products of both of these materials are effective against Victoria blight of oats (1). Agrox and Anticarie also are effective as standard treatments against wheat bunt at ½ oz. to the bushel. Since these compounds are only slightly volatile, their fungicidal properties presumably depend upon direct contact. CP hexachlorobenzene, though presumably nonvolatile, has been shown to be toxic to spores of *T. caries* in the absence of direct contact (6). It was included in this study as a standard for evaluating the performance of Anticarie.

The varieties of wheat used were Elgin, a winter wheat, and Red Bobs, a spring wheat. These were inoculated with 0.5 g of spores of race T-5 (5) per 100 g of seed. The medium used for spore germination studies was Difco 2 per cent water agar.

The methods and procedures were designed to determine the effectiveness of the vapors of these fungicides in preventing spore germination on an agar medium and in preventing infection by seed-borne spores. For the spore-germination tests on agar, the fungicides were placed in individual small vials (7 mm deep and 20 mm in diameter), and each vial was set on the surface of 2 per cent water agar in separate petri plates. A suspension of spores was spread over the remaining surface of the agar, and the plates were closed and sealed with rubber-band petri-plate seals. The plates were then incubated at 15°C. Observations on spore germination were begun after 5 days' incubation and continued at 1-day intervals for 10 days. Separate plates were used for each day's observation and were discarded after germination percentages were taken.

In the study on infection by seed-borne spores, the vials containing the fungicide were placed in empty petri plates and the inoculated seed (5 cc) was distributed around the vial in each plate. These plates also were sealed with rubber-band petri-plate seals and placed at 15°C. All of the inoculated seed from 1 plate was removed and planted, either in the field or in the greenhouse, at intervals of 24 hours for 14 days, beginning after the first 24 hours of exposure.

The fungicides were tested at 2 rates. The lower rate used was based on the report of Siang and Holton (6) that 10 mg of CP hexachlorobenzene inhibited germination of spores of *Tilletia* spp. on agar. The higher rate was based on the amount of fungicide used to treat 250 cc of wheat seed at the rate of ½ oz. per bushel. This rate exposed the inoculated seed used in this test to approximately 50 times the amount of these fungicides carried by wheat seed treated commercially at the recommended rates of

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[2] Pathologists, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Pullman, Washington.

TABLE 1.—*Percentage germination of spores of Tilletia caries on water agar exposed to vapors emanating from 3 seed-treatment fungicides*

Fungicide applied		Percentage germination after exposure to vapors for indicated periods			
Kind	Amount	5 Days	6 Days	8 Days	10 Days
None	0	51	52	46	66
Anticarie	20 mg	0	0	0	0
Ceresan M	20 mg	75	76	77	49
Panogen 15	0.1 ml[a]	T[b]	2	4	2
Anticarie	120 mg	0	0	0	0
Ceresan M	120 mg	61	32	39	42
Panogen 15	0.75 ml[a]	1	0	T	0

[a] Amount of a 1:4.5 dilution of Panogen 15.
[b] Less than 0.5 per cent.

application. Panogen 15, a liquid, was used as a 1:4.5 water dilution. The other materials were used as dry powders. The field tests on wheat bunt were made with Elgin and Red Bobs seeded in the fall and spring, respectively, whereas Red Bobs was used for the greenhouse tests.

Spore germination percentages were determined by counting a total of 200 spores in 2 locations on the agar, and bunt percentages were based on total head counts.

EXPERIMENTAL RESULTS.—The results from spore germination tests are summarized in Table 1. These data show that the vapor action of Panogen 15 and Anticarie was highly effective in preventing spore germination at both rates of application. No germination occurred in the Anticarie plates, and only small percentages occurred in the Panogen 15 plates. In contrast, the vapor action of Ceresan M was completely ineffective at the lower rate and possibly only slightly effective at the higher rate after exposure for 6 days or longer. It is also apparent from the data in Table 1 that periods of exposure longer than the minimum time required for spore germination in the check plates did not appreciably increase the effectiveness of the vapor action against spore germination. The results with CP hexachlorobenzene were comparable with those for Anticarie and confirm the report by Siang and Holton (6). The performance of Agrox in this study was identical with that of Ceresan M. Therefore, CP hexachlorobenzene and Agrox are not included in the tabular summaries.

A portion of the results from tests to determine the effectiveness of fungicidal vapors in controlling bunt originating from infection by seed-borne spores is summarized in Table 2. These data show that only Panogen 15 controlled wheat bunt by vapor action. It was highly effective at both rates of application in 2 of the 3 tests with wheat. The lesser effectiveness exhibited in the field test with spring wheat (Table 2) probably was due to experimental error that allowed some vapor to escape during the exposure period, thereby reducing its total effect.

Vapors of both Ceresan M and Anticarie were totally ineffective against bunt at the low rate, and Ceresan M was only slightly effective at the higher rate after exposure for at least 5 days. The results with Agrox and CP hexachlorobenzene were comparable with those for Ceresan M and Anticarie. Apparently all of these materials require direct contact

TABLE 2.—*Percentage of smutted heads in wheat plants grown from seed inoculated with race T-5 of Tilletia caries and exposed to the volatile products of 3 different seed-treatment fungicides*

Fungicide applied		Percentage of smutted heads after exposure of seed to vapors for indicated period					
Kind	Amount	1 Day	3 Days	5 Days	7 Days	9 Days	14 Days
		Spring wheat (Greenhouse)					
None	0	86	91	97	100	83	77
Anticarie	20 mg	79	81	95	98	70	87
Ceresan M	20 mg	56	92	85	95	86	78
Panogen 15	0.1 ml	2	1	0	0	0	0
Anticarie	120 mg	56	97	95	92	94	65
Ceresan M	120 mg	65	92	32	17	28	14
Panogen 15	0.75 ml[a]	2	0	0	0	0	0
		Spring wheat (field)					
None	0	72	92	82	58	40	66
Anticarie	20 mg	86	94	93	83	50	76
Ceresan M	20 mg	81	93	84	67	28	48
Panogen 15	0.1 ml	61	22	25	4	2	8
Anticarie	120 mg	82	97	89	76	40	78
Ceresan M	120 mg	81	71	80	10	29	17
Panogen 15	0.75 ml[a]	60	0	1	0	0	0
		Winter wheat (field)					
None	0	70	79	63	88	57	22
Anticarie	20 mg	68	68	75	76	75	68
Ceresan M	20 mg	84	76	71	87	67	6
Panogen 15	0.1 ml[a]	5	2	0	2	3	0
Anticarie	120 mg	86	82	68	58	76	9
Ceresan M	120 mg	84	75	70	52	38	39
Panogen 15	0.75 ml[a]	2	0	1	0	0	0

[a] Amount of a 1:4.5 dilution of Panogen 15.

for effective toxic action against dry seed-borne spores. Worthy of note in this connection is the contrasting action of the hexachlorobenzene preparations against spore germination in culture and on dry wheat seed. This differential performance may be explained on the basis of a toxic action that is effective only against spores in a state of germination, which occurs only in the presence of moisture. Elucidation of this point requires further study.

Almost all plants in rows planted from seed exposed to the vapors of Panogen 15 were taller than those in check rows and in rows planted from seed exposed to the vapors of the other fungicides. This was particularly noticeable in the winter wheat test in which the plants from seed exposed to Panogen 15 were 3–6 in. taller than those grown in adjacent rows from seed exposed to other treatments. This difference in plant vigor was believed due to the control of bunt by Panogen 15 rather than to a stimulating effect of the treatment.

REGIONAL SMUT RESEARCH LABORATORY
WASHINGTON AGRICULTURAL EXPERIMENT STATIONS
PULLMAN, WASHINGTON

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SCREENING FUNGICIDES FOR CONTROLLING DAMPING-OFF OF TREE SEEDLINGS [1]

Olli Vaartaja

SUMMARY

The control given by 109 chemicals against *Rhizoctonia solani* and *Pythium debaryanum* was tested with pine and birch seeds in petri dishes. Most of the chemicals were phytotoxic or failed in control. Rimocidin, captan, and thiram, at a wide range of concentrations, controlled the diseases without marked phytotoxicity. Nineteen chemicals performed well at narrower concentration ranges. Thirteen chemicals were effective against Pythium but not against Rhizoctonia.

Tree seedlings grown in forest nurseries may suffer severely from damping-off. Amongst the factors tending to favor the disease are heavy fertilizing, shading, and watering. These may disturb the balance of soil microflora, which under natural conditions would have a buffering action against damping-off epidemics. In addition, the seedlings are grown close together in dense stands, a situation that facilitates the spread of disease. Furthermore, the initial development of most tree seedlings is very slow, and a long susceptible stage exists. Ordinary seed treatment does not remain effective very long (6, 15) and therefore usually does not control postemergent damping-off. Similarly, sterilized soil tends to become reinfested before the danger period is over. Two methods may be tried to overcome these difficulties: 1) repetition of seedbed applications and 2) seed pelleting with an extra amount of fungicide bound with special stickers such as methyl cellulose. To be successful, the fungicide or chemotherapeutant must have a very low phytotoxicity.

A screening method was developed for testing chemicals in petri dishes for the control of damping-off without harm to seeds and seedlings. The chemicals were tested at a wide range of concentrations. Those with a large difference between *dosis curativa* and *dosis tolerata* are being investigated further in soil tests in pots and in the field.

METHODS.—An isolate of *Rhizoctonia solani* Kühn and an isolate of *Pythium debaryanum* Hesse were used as test pathogens. The *Rhizoctonia* isolate proved to be highly pathogenic to species and strains of *Pinus*, *Picea*, and *Caragana*. This isolate readily

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produced basidiospores on certain media in petri dishes and appeared to belong to the species *Corticium praticola* Kotila. It was commonly isolated from dying seedlings of several tree species in various nurseries in Saskatchewan. *P. debaryanum* also is common in Saskatchewan, and the isolate showed high virulence on several tree species in laboratory tests.

The fungi were grown in petri dishes in 30 ml of half-strength Difco corn-meal agar. A thin colony was formed over the whole agar surface within a few days. Then 20 ml of melted agar (at 40° C), mixed with the test chemical, was poured on the colony, and a solid top layer about 3 mm thick was formed. Fifty seeds of *Pinus banksiana* Lamb. were sown on this layer; a number of the chemicals were tested also with seeds of *Betula verrucosa* Ehr. The dishes were kept at room temperature under continuous illumination of 100–200 foot candles for 14 days, and the survival was recorded. The 109 chemicals chosen[2] were tested at several concentrations in a 10-fold dilution series. The concentrations of the chemicals given in tables were based on weights of the fungicide preparations (not the "active ingredient") and of the top layer of agar before volatilization, diffusion, or drying took place. If a chemical appeared effective, the test was repeated 2–6 times. For the most promising chemicals, the tests were repeated 4–10 times.

Some chemicals encouraged the growth of Trichoderma contaminants. In general, contamination was avoided by using seed of good quality, by detecting and discarding empty or diseased seeds, and by growing the stock cultures of the test fungi on rose bengal: streptomycin agar to suppress bacteria. In some cases the surfaces of seeds were sterilized with calcium hypochlorite and alcohol, but this was unnecessary for routine tests.

RESULTS.—In most dishes, either the seeds did not germinate or the seedlings soon died. Some dishes showed a clear contrast to this, having 30–45 well-developed seedlings. In such cases the chemical was effective against the fungus without injury to the seedling, and the disease was recorded as being controlled. If the number of well-developed seedlings was 10–30, partial control was recorded.

Although an exact comparison of the chemicals was not possible on the basis of this coarse screening, the tested materials were divided into 5 groups according to their performances. Results with the 2 best groups, D and E, are given in Tables 1 and 2. The fungicides containing captan, Rimocidin, or thiram (Table 2) were the only fungicides that consistently performed well at more than 1 concentration tested.

TABLE 1.—*Group D. Chemicals that controlled Pythium debaryanum (but not Rhizoctonia solani) without injury to Pinus banksiana at concentrations indicated*

Chemical	Effective concentration[a] (ppm)
B-856 C(75% 1,3-dichloro-5,5-diphenylhydantoin)	50,500
Bordeaux mixture	50,500[b]
Boric Acid	500[b]
Chinosol (8-quinolinol sulfate)	50[b]
Cinnamic acid	5,50
Copper sulfate	5
Dodecyl sodium sulfate	500[b]
Gladiolic acid (antibiotic)	50[b]
NP-1083 (25% 1-fluor-2,4-dinitrobenzene)	50
Phenyl hydrazine	50
8-Quinolinol sulfamate	5,50
Semesan Bel (2-chloro-4-(hydroxymercuri) phenol and isomers; 4-(hydroxymercuri)-2-nitrophenol and isomers)	5000[c]
Sulfuric acid	500

[a] Lower concentrations were ineffective and higher ones phytotoxic.
[b] Tested also with *Betula verrucosa* with similar results.
[c] Phytotoxic to *B. verrucosa*.

The chemicals found ineffective against *R. solani* and *P. debaryanum* or effective only at concentrations toxic to *P. banksiana* were the following (group A): Acetic acid, 1-acetyl-2-thiohydantoin, acridine orange, allyl alcohol, ammonium sulfamate, ammonium thiocyanate, Auramine O, benzene hexachloride, benzotriazole, benzyl salicylate, bismuth salicylate, 2-carboxymethyl mercaptobenzothiazole, chloromycetin (chloramphenicol), Crag 658 (95 per cent copper zinc chromate ($15\ CuO \cdot 10\ ZnO \cdot 6\ CrO_3 \cdot 25\ H_2O$)), Crag 974 (95 per cent tetrahydro-3,5-dimethyl-2*H*-1,3,5-thiadiazine-2-thione), D.D.T., 2,4-D, dextrose, 4,6-dinitro-cresol, Dowicide G (75 per cent sodium pentachlorophenoxide), ethylene glycol, fast green, formaldehyde, 1-fluoro-2,4-dinitrobenzene, gallic acid, indole, lactic acid, maleic hydrazide, methylene blue, methyl salicylate, naphthalene acetic acid, Nile blue, phenol, phenothiazine, phthalic acid, Phygon (50 per cent 2,3-dichloro-1,4-naphthoquinone), picric acid, potassium nitrate, pyroligneous acid, rose bengal, salicylic acid, Stauffer N-244 (3-(*p*-chlorophenyl)-5-methylrhodanine), Stauffer N-521 (the same active ingredient as Crag 974), streptomycin sulfate, subtilin (antibiotic), succinic acid, sulfadiazine, sulfanilic acid, sulfapyridine, tannic acid, thiosemicarbazide, thiourea, 2,4,6-trinitrobenzoic acid, and triphenyl-2*H*-tetrazolium chloride.

The chemicals that gave partial control against *R. solani* or *P. debaryanum* or both at some of the concentrations used were as follows (group B): Acridone, ascorbic acid, benzoic acid, *p*-dichlorobenzine, Karathane WD (25 per cent 2-(1-methylheptyl)-4,6-dinitrophenyl crotonate and isomers), Lo 738 (75 per cent ethylenebisthiuram trisulfide), Lysol (mixture of crude cresols), PCNB (75 per cent pentachloronitrobenzene),

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TABLE 2.—*Group E. Chemicals that controlled Pythium debaryanum and Rhizoctonia solani without injury to Pinus banksiana at concentrations indicated*

Chemical	Concentrations[a] that gave control of Pythium	Rhizoctonia
	ppm	ppm
Arasan (75% thiram[b])	50–5000	50–5000
Captan (75% captan[c])	50–5000	50–5000
	(50)	(50)
Dibenzothiophene	5000	5000
Dithane D-14 (19% nabam[d])	500	500
Dithane Z-78 (65% zineb[e])	50–5000	500
	(50)	
Fermate (76% ferbam[f])	**5–5000**	500
Fluorene	5000	5000
Heptachlor 2E[g]	5000	5000
Lindane (γ-1,2,3,4,5,6-hexachlorocyclohexene)		5000
Malachite green[h]	**50**	50
Manzate (70% maneb[i])	**50–500**	**500**
Mercuric chloride	50	50
Orthocide 75 (75% captan[c])	50–5000	50–5000
Panogen (2% cyano(methylmercuri) guanidine)	50	50
Parzate (65% zineb[e])	500	500
Rimocidin	**50–500**	**50–500**
Spergon (96% chloranil[j])	500	**500**
Tersan (75% thiram[b])	**50–5000**	50–5000
Thiolutin (antibiotic)	No test	**50**
Thioneb 5QW (50% polyethylene thiuram sulfide)	5000	5000
Trinitrotoluene	5000	5000
Vancide 51[k]	**500**	500
Zinc chloride	500	500
XSF (a silver preparate)	No test	5000

[a] Lower concentrations were ineffective and higher ones were phytotoxic. Those in parentheses were tested with *Betula verrucosa;* those in bold face were tested with *Pinus banksiana* and *B. verrucosa* with similar results.
[b] Bis(dimethylthiocarbamoyl) disulfide.
[c] *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide.
[d] Disodium ethylenebis[dithiocarbamate].
[e] Zinc ethylenebis[dithiocarbamate].
[f] Ferric dimethyldithiocarbamate.
[g] 23% 1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene.
[h] Oxidation product of 4,4′-benzylidenbis[*N*,*N*-dimethylaniline].
[i] Manganous ethylenebis[dithiocarbamate].
[j] Tetrachloro-*p*-benzoquinone.
[k] Sodium dimethyldithiocarbamate and sodium derivate of 2-mercaptobenzathiazole; 30 per cent total active ingredients.

Perenox (cuprous oxide; 50 per cent copper), *o*-phenylphenol, saccharin (*o*-benzoic sulfimide), sodium selenate, Tri-Cop (basic copper sulfate; 50 per cent copper), 10-undecanoic acid, Ustilagic acid (antibiotic), and xylol. About half of these chemicals listed were also tried with *B. verrucosa*, but none performed well. In contrast, some chemicals were ineffective or phytotoxic with *P. banksiana* but gave good control with *B. verrucosa*. These were (group C): Acti-dione (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide) at 50 and 500 ppm, against both pathogens; Fradicin (antibiotic) at 0.005 and 0.5 ppm, against Pythium; Helixin B (antibiotic) at 5 ppm, against Rhizoctonia (not tested with Pythium); Merck 53 (antibiotic) at 500 ppm, against Rhizoctonia (not tested with Pythium); quinine sulfate at 0.5 ppm, against Pythium; and Gladiolic acid (antibiotic) at 0.005–50 ppm against Pythium.

The distinctly good performances of the 3 chemicals in group E indicated that further study of them is justified. The majority of the chemicals tested did not fill the high requirements of the test. The relative effectiveness of a number of chemicals in the intermediate classes was somewhat uncertain. With these chemicals, the balance between the host and pathogen was delicate and possibly was influenced by an environmental change. Reduction of illumination from 150 to 50 foot candles increased mortality in certain replications. Variation in temperature also encouraged the disease through top infection: the fungus grew on the lids of petri dishes that were wet from condensation water. On the whole however, the results were consistent. In the few variable cases, results were interpreted with caution.

The screening gave in general the same results with both species of test trees. Most of the chemicals gave similar control against both test fungi (Table 2), but a number of the chemicals (Table 1) were much more effective against Pythium than against Rhizoctonia.

DISCUSSION.—A large number of chemicals have been reported by various investigators to act chemotherapeutically upon various plant diseases. In the present investigation, chemicals of this type gave negative results with the exception of fluorene and dibenzothiophene (Table 2). Further experiments showed that these 2 chemicals obviously penetrate pine seedlings and act systemically against Rhizoctonia (17). These and related compounds have been shown to be chemotherapeutants against Fusarium wilt of tomato (5). Few chemicals are expected to be chemotherapeutants against *R. solani* and *P. debaryanum*, since these pathogens are very virulent and little specialized. The insecticides Lindane and Heptachlor 2E possibly acted chemotherapeutically, although Lindane also has some fungicidal properties (14). Heptachlor 2E is reported as a chemotherapeutant against nematodes (8) and is known to possess low phytoxicity.

Although pentachloronitrobenzene (PCNB), Crag 974, and Stauffer N-521 are reported to be specifically effective fungicides against *R. solani* (9), they, as well as many other fungicides, failed in the present tests (groups A and B). Peculiarly, PCNB gave good protection to the roots; the disease always began in the tops of the seedlings when this chemical was used. This material apparently lacks systemic properties.

The different reactions of Pythium and Rhizoctonia to a number of chemicals (Table 1) may be an explanation of the variability of the performances of fungicides in the field.

Trichoderma, sp. when present as a contaminant, increased the survival of seedlings in tests with trinitrotoluene. This fungus is relatively tolerant also

to carbon disulfide (4), fluorides (10), and formaldehyde (12). Use of selective chemicals and seeding with nonpathogenic saprophytes would combine biological and chemical control. The success of thiram may be based in part on such dual influence in soil (13, 16). To study this possibility further, dishes with thiram, captan, and Rimocidin from the above tests were seeded with a mixture of saprophytic soil bacteria. Certain bacterial colonies developed well when the concentration of the chemical was 500 ppm, high enough to inhibit the pathogens. Thus, 3 of the best chemicals of this screening showed useful selectivity in their action.

Many fungicides based on the toxicity of metallic ions gave good, but not excellent, results. The best in this group appeared to be Panogen, which has given good control of damping-off in various plants in nursery flats (1).

Fungicides based on dithiocarbamates and related compounds (thiram, ferbam, nabam, maneb, zineb, Vancide 51) are increasing in importance in controlling various plant diseases. These compounds were among the best in this screening (Tables 1, 2); however, there was variability in the performances of some of this group. A possible explanation of this variability may be the finding of Ludwig and Thorn (11) that the active principles of most of these fungicides are breakdown products of the applied materials, and the decomposition often is affected by various factors such as pH.

The best chemical of the test, Rimocidin, is a highly fungicidal antibiotic from *Streptomyces rimous*. Rimocidin has also controlled Rhizoctonia disease of potato, tomato, and lettuce (7).

Only 3 out of 109 chemicals consistently performed well at more than 1 concentration. Many of the others may give satisfactory control under certain conditions within a limited concentration range. When the variation in conditions and the susceptibility of tree seedlings to chemical injury (3, 18) are considered, the safest chemicals should be preferred for further trials in the field. In seed-pelleting trials by Berbee et al (2) in Wisconsin, thiram has already given promising results, whereas many other fungicides failed. Vaartaja and Wilner (unpublished) found pelleting with thiram, captan, and zineb effective in a Saskatchewan nursery. Captan and thiram have performed well in the repeated seedbed applications by Strong (15) in Michigan and by Vaartaja (unpublished) in Saskatchewan. More field trials under various conditions are needed, however, before the field performance of these fungicides in seedbeds can be predicted with safety.

FOREST PATHOLOGY LABORATORY
SASKATOON
SASKATCHEWAN

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DAMPING-OFF PATHOGENS OF CONIFERS AND OF CARAGANA IN SASKATCHEWAN [1]

O. Vaartaja and W. H. Cram

SUMMARY

Many organisms were isolated from diseased seedlings at Saskatchewan forest nurseries. The most numerous isolates were species of *Fusarium*, *Pythium*, *Rhizoctonia*, *Alternaria*, *Phoma*, and *Cylindrocarpon*. Jack pine and caragana seedlings in sterile test tubes were inoculated with the isolates. In these tubes, most isolates of *Rhizoctonia* and *Pythium* killed all seedlings in a few days. Isolates of *Alternaria*, of *Phoma*, of *Cylindrocarpon*, of 8 species of *Fusarium*, and of many other fungal genera were less pathogenic. Under conditions favoring pathogenicity, various degrees of pathogenic potentials were represented by the fungal isolates.

Isolates of different fungal genera and a nematode were used as inocula in greenhouse tests with several tree species and progenies in nonsterilized nursery soil. Species and progenies responded differently to the inocula. *R. solani* reduced the average emergence of caragana from 95 to 50 per cent and of Scots pine from 99 to 73 per cent. Postemergent losses were 68 and 64 per cent, respectively. *R. solani* did not reduce emergence of any of the spruces but increased emergence of 1 of the white spruce progenies. *R. solani* caused significant postemergent losses in one of the 2 progenies of each of Colorado spruce and white spruce. *Pythium* (*debaryanum?*) was slightly pathogenic to seedlings of all trees and significantly so to those of 1 white spruce progeny. *Fusarium oxysporum* var. *redolens* seemed to be mildly pathogenic to seedlings of 1 Colorado spruce progeny. *Alternaria* (*tenuis?*) and a nematode (*Panagrolaimus* sp.) were not pathogenic in this test.

Fusarium species were commonly isolated from roots of the surviving, apparently healthy seedlings. Possibly these fungi were parasitic but only weakly pathogenic. A "mixed" inoculum caused less mortality than did Rhizoctonia alone. This suggests useful antagonism among some constituents of the inoculum. Microbiological antagonism was considered also to have influenced the decreased virulence of the inoculum fungi in nonsterilized soil as compared to their virulence in tests conducted in sterilized test tubes.

The pathogenic potentials of *Rhizoctonia solani* Kühn, *Pythium debaryanum* Hesse, *P. ultimum* Trow, *P. aphanidermatum* (Edson) Fitzp., *P. irregulare* Buis., *Phytophthora* spp., *Fusarium* spp., and some other fungi on various tree seedlings have been established in inoculation studies (4, 6, 8, 11, 17, 26, 27, 29, 34, 35, 37). Virulence of strains of *R. solani* varies greatly (6, 11, 12, 17) and those of *P. debaryanum* considerably (11, 31). In certain experiments, some strains of *Fusarium* were not pathogenic (4, 11, 14, 17, 27, 34). Although most of the inoculations were made in sterilized soil or soil other than that of a nursery, there is little doubt of the great importance of *Rhizoctonia* and *Pythium* species as universal damping-off pathogens in forest nurseries. After the pathogenicity of a soil organism has been determined by aseptic methods following Koch's postulates, however, the pathogen should also be studied in nonsterilized soil. As Hartley (11), Sanford (21, 22), and Garret (9) have emphasized, this type of study is needed to ascertain the importance of a soil organism in natural environment. The occurrence of damping-off in controls has caused investigators to avoid the use of naturally infested soils (34). The factors influencing damping-off have rarely been studied with the exception of damping-off by *R. solani* and by *P. irregulare* in conifer beds in Wisconsin (18, 19). Inoculation studies are lacking for some important species, such as caragana (*Caragana arborescens* Lam.), although *Phytophthora cinnamomi* Rands, *R. solani*, and *Fusarium* spp. have been isolated from caragana seedlings (35).

In the Canadian Prairie Provinces, the forest nurseries are located in the area at the borders of naturally occurring forests or outside of these. The seedbeds of these nurseries are mostly on alkaline soil. Under these conditions, serious damping-off has occurred.

All conifers grown in the nurseries are susceptible, namely *Picea glauca* (Moench) Voss, *P. pungens* Engelm., *P. engelmannii* Parry, *P. abies* (L.) Karst., *Pinus banksiana* Lamb., *P. sylvestris* L., *P. contorta* var. *latifolia* Engelm., *P. resinosa* Ait., and *Larix sibirica* Lebed. Similarly, an important broadleaved species, *Caragana arborescens*, is frequently diseased. The extent of seedling and seed losses varies greatly. Postemergent losses of Colorado spruce (*P. pungens*) have ranged from 1 to 56 per cent in the 1947 to 1952 period at the Indian Head Nursery. Alarming epidemics were reported (7) for 1953, when pre-emergent and postemergent mortalities reached 68 and 88 per cent, respectively.

Confronted by this situation, the authors began a study of the etiology of these epidemics. At first a large number of isolations were made from diseased seedlings, and the pathogenic potentials were determined in test tubes for a number of organisms isolated. The actual importance of various representative organisms was then explored in tests with nonsterilized

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seedbed soil.

METHODS.—Thousands of isolations were made from diseased seedlings of various species grown in 1953–1955 in various forest nurseries in the Canadian Prairie Provinces, mainly Saskatchewan. The seedlings usually were treated with a water solution of 0.5 per cent $HgCl_2$ and 50 per cent ethanol and plated on 1.0 per cent agar dishes.

Representatives of the isolates were tested by the following method. Test tubes containing 60 ml of agar medium were seeded by inserting a piece ($4 \times 4 \times 4$ mm) of a fresh malt-agar culture. The medium contained KH_2PO_4, 0.25 g; $CaCl_2$, 0.025 g; $NaCl_2$, 0.012 g; $MgSO_4$, $7H_2O$, 0.07 g; NH_4NO_3, 0.10 g; agar, 12 g; and tap water, 1000 ml. Five germinating seeds of jack pine (*P. banksiana*) or of caragana (*C. arboescens*) were transferred to each tube from malt-agar plates, where the seeds that were contaminated despite surface sterilization could be detected and discarded. The medium provided healthy and moderately vigorous growth of inoculated seedlings for at least 180 days. The fungus colonies in the tubes were thin but capable of parasitism. Fairly low light intensity was used, as many mildly virulent fungi can kill tree seedlings only under low light intensity (28). The less prevalent isolates were tested twice, and further replications were made of the more common isolates. Flora in the tubes were examined at the conclusion of each test. Penicillium or bacteria contaminations were detected in a few of those tubes in which seedlings survived. Such cases were retested or the data rejected; the same was done for tests where replications gave divergent results.

Seeds were collected from 2 trees each of white spruce, Colorado spruce, Scots pine, and caragana grown at Indian Head, Saskatchewan. The seeds were standardized by screening, dipped in 0.5 per cent mercuric chloride solution, and rinsed with water. Spruce seed was then stratified for 20 days and pine and caragana seed for 13 days in moist sterilized sand at 5°C. Seed was sown in flats in a 2:1 mixture of seedbed soil and sand. The soil was slightly alkaline loam from the Indian Head forest nursery.

Cultures grown on potato-dextrose agar were finely divided and suspended in water. This suspension was sprinkled on the flats. The different inocula were 1) *Rhizoctonia* (*Corticium praticola*), isolate No. 1143 from caragana; 2) *Pythium* (*debaryanum ?*) isolate No. 1054 from white spruce; 3) *Fusarium oxysporum* var. *redolens*, isolate No. 1130 from caragana; 4) *Alternaria* (*tenuis ?*), isolate No. 1120a from Siberian larch; 5) a nematode (*Panagrolaimus* sp.), isolated from Scots pine, and an associated bacterium; 6) a mixture of each of the above; and 7) the control—instead of an inoculum, a drench of Tersan (50 per cent bis(dimethyldithiocarbamoyl) disulfide) was sprinkled twice on the flats (2×100 lb./acre). This check treatment largely controlled the disease otherwise caused by the natural soil inoculum. Inocula 1–4 were selected as very virulent representatives amongst the fungi used in the test-tube studies. The number of living particles in the various inocula varied from 10^5 (nematode) to 10^9 (Fusarium) per flat. The tree species and treatments were randomized in a split-plot design with 3 replications.

The effect of the inocula on each other was studied by plating them in pairs in all combinations on various agar media; none prevented the growth of any of the others except the bacterium associated with *Panagrolaimus* sp., which inhibited most of the growth of Pythium and temporarily reduced the growth of Rhizoctonia.

The soil medium was maintained at an available moisture content of 80–90 per cent as indicated by the Bouyoucos (2) block method; tap water was applied as required, twice daily. Temperature in the greenhouse varied between 11° and 18°C at night and between 18° and 30°C in the daytime. All the inoculum fungi grew well at these temperatures. As the test was conducted in December and January, additional lights were used to provide photoperiods of 16 hours.

Germination and postemergent mortality were recorded daily. Reisolation of the inoculum organisms was attempted from the majority of damped-off and surviving seedlings.

ISOLATIONS.—The most prevalent genus in isolations from diseased seedlings was *Fusarium;* the species of this genus most frequently isolated were *F. oxysporum* Schlecht. emend. Snyd. & Hans., *F. acuminatum* Ell. & Ev., and *F. solani* (Appel & Wr.) Whet. emend. Snyd & Hans.[2]. Fusaria often occurred in association with *Pythium ultimum*, *P. debaryanum*[3], and *Rhizoctonia solani*. One or more of these 3 have been isolated in all epidemics studied. In certain sudden epidemics, nearly all the isolates were *Pythium*. Plating of 151 coniferous seedlings from Big River Nursery in 1953 gave the following isolate percentages: *Pythium*, 61; *Fusarium*, 6; *Alternaria*, 3; *Stemphylium*, 3; and miscellaneous genera, 4. Twenty-three per cent of the tissues were sterile. Many of the isolates of *R. solani* readily produced basidiospores and were considered to belong to the species *Corticium praticola* Kotila.

Alternaria spp., *Phoma* spp. (mostly *P. glomerata* (Cda.) Wr. & Hochl.), and *Cylindrocarpon* spp. also were commonly isolated, but these usually were associated with other fungi. Species of *Alternaria* tended to occur on the top part of the seedlings. Some of the fungi occasionally isolated are included in Tables 1 and 2. Other isolates were *Pythium vexans* d By., *Stemphylium* sp., *Torula* spp., *Monilia* sp., *Geotrichum* sp., *Penicillium* sp., *Chaetomium* sp., *Mucor* sp., *Sclerotinia* sp., and a number of unidentified fungi. *Phytophthora cactorum* was isolated only from Scots pine (*P. sylvestris*) at Indian Head in 1954 and 1955. The isolates of *Aphanomyces* were close to the species *A. cochliodes* Drechs. and *A. euteiches* Drechs. These

[2] Many of the *Fusarium* isolates were identified by W. L. Gordon, Winnipeg, Manitoba.

[3] Some of the *Pythium* isolates were identified by J. T. Middleton, Riverside, California.

TABLE 1.—*Survival of seedlings of Pinus banksiana following inoculations, in test tubes under sterile conditions, with various organisms*[a]

Isolate	Organism	No. of isolates tested	No. of replications	Days of survival[b]
	Pythium ultimum and *P. debaryanum*	7	32	4
1080	*Rhizoctonia solani* (sterile strain)	1	6	11
	Corticium praticola	4	13	12
1112	Unidentified (sterile)	1	5	11
1552y	*Botrytis cinerea* Pers.	1	2	12
1722	*Phytophthora cactorum* (Leb. & Cohn.) Schroet.	1	2	13
1120a	*Alternaria tenuis* Auct. *sensu* Wiltshire	1	3	15
1089	*Alternaria tenuis*	1	2	16
1124	*Alternaria* sp.	1	4	17
1809	*Fusarium solani*	1	2	23
1132	*Trichoderma viride* Pers. *ex* Fr.	1	5	28
1130	*F. oxysporum* var. *redolens*	1	2	90
1431	*Cephalosporium* sp.	1	2	120
	Fusarium spp.[c]	19	38	180[d]
	Alternaria spp.	11	25	180[d]
	Cylindrocarpon sp.	2	4	180[d]
1052	*Phoma glomerata*	1	10	180[d]
1708	*Aphanomyces* sp.	1	2	180[d]
	Rhizoctonia sp.	2	4	180[e]
1579B	*Hormodendron* sp.	1	2	180[e]
	F. acuminatum	1	2	180
	F. oxysporum, mutant	1	2	180
	Dematium pullulans d By.	2	9	180
	Panagrolaimus sp.	1	6	180
	Amoeba	1	2	180
	None (check)		14	180

[a] Under 250 foot candles of light at 20–23°C.

[b] Survival time was recorded when the last of 5 seedlings in a tube died or in 180 days when the test was terminated. Averages of these recordings are given for each inoculum species.

[c] *F. oxysporum*, *F. oxysporum* var. *redolens* (Wr.) Gordon, *F. solani*, *F. acuminatum*, *F. equiseti* (Cda.) Sacc., *F. moniliforme* Sheldon, emend. Snyd. & Hans., *F. sambucinum* Fckl., *F. culmorum* (W. G. Sm.) Sacc.

[d] Most but not all of 5 seedlings died.

[e] Killed or deformed roots.

isolates were obtained only from *P. glauca* at Wasagaming, Manitoba, in 1954.

Bacteria were usually present but seldom alone. Amoebae, paramoeciae, mites, and nematodes (at least 3 species) occurred occasionally. The incidence of a nematode was once as high as 21 per cent in 220 platings from Scots pine (29). This nematode was identified by Dr. A. D. Baker as *Panagrolaimus* sp.

Attempts were made to isolate fungi from apparently healthy seedlings by the use of the same surface-sterilization technique as that used for diseased tissues. In general, no fungi were obtained from juvenile tissues, but older tissues often yielded various fungi. For instance, the incidence of fungi obtained from 20 pieces of stem from 2-year-old *Pinus sylvestris* were *Alternaria*, 16; *Phoma*, 12; *Fusarium*, 2; *Rhizoctonia*, 1; *Cylindrocarpon*, 1; *Dematium pullulans*, 1. Species of *Pythium* and of *Rhizoctonia* rarely were isolated from such seedlings, probably only in cases of incipient disease.

POTENTIAL PATHOGENICITY.—The time required for the fungi to kill the seedlings varied greatly (Tables 1, 2). No pathogenicity was demonstrated by an amoeba, by a nematode, nor by 5 of the 85 fungal isolates. These may not lack pathogenic potentials, however, for they may be wound parasites or specialized to certain other hosts. A number of fungi showed only slight pathogenicity; perhaps they are only saprophytes under most conditions. Under the test conditions, there were no clear-cut differences among virulent pathogens, weak pathogens, and those fungi normally not considered as pathogens. The snow mold fungus, which was tested as an extra control, demonstrated some pathogenic potential on the experimental seedlings but less so than the least virulent damping-off associate.

The wide difference in results between 2 strains of *Trichoderma viride* (No. 1132 and 820, Table 2) is noteworthy as contrasted to the uniformity of those of *Pythium;* the standard error was only ± 0.2 (mean = 4) in 32 tests with the species of *Pythium* listed in Table 1. Although the 2 isolates of *T. viride* were morphologically similar, isolate No. 820 smelled of mushrooms, whereas isolate No. 1132 had a strong anis-like odor.

The fungi killed pine seedlings more rapidly than they killed caragana seedlings (Table 2); nevertheless, with a few exceptions, the virulence of the fungi on both hosts was of the same order. In experiments similar to those in Tables 1 and 2, the pathogenic

TABLE 2.—*Survival of seedlings of Pinus banksiana and of Caragana arborescens following inoculations, in test tubes under sterile conditions, with various organisms*[a]

Isolate	Organism	Days of survival	
		Pinus	Caragana
1698	*Pythium pulchrum* von Minden	5	6
1347	*R. solani* (*C. praticola*)	10	3
1341	*Phytophthora cactorum*	10	19
1120a	*Alternaria tenuis* ?	10	19
1028	*Alternaria tenuis*	13	35
1350	*Gliocladium roseum* Bain	14	60
1022	*Phoma* sp.	15	37
1195	*Sclerotium* sp.	15	54
1132	*Trichoderma viride*	16	57
1407	*Alternaria* sp.	21	40
1377	*Cylindrocarpon radicicola* Wr.	21	44
1403	*Phoma glomerata*	24	40
999	*Epicoccum purpurascens* Ehr.	24	57
891	*Papularia* sp.	30	48
806	*Phoma* sp.	33	38
973	*Rosellinia* sp.	40	66
765	*Aspergillus* sp.	42	55
983	*Pestalotia* sp.	52	105
1364	A bacterium	60	[b]
903	*Nigrospora* sp.	63	49
1382	*Streptomyces* sp.	95	[b]
820	*Trichoderma viride*	103	120
1020	A snow mold fungus (Check I)	111	114
	None (Check II)	180	180

[a] Under 150 foot candles of light at 23°C.

[b] Not recorded.

TABLE 3.—*Germination percentage*[a] *of seed from 2 trees each of Picea glauca, P. pungens, Pinus sylvestris, and Caragana arborescens in tests with nonsterilized soil*

Inoculum	*P. glauca*		*P. pungens*		*C. arborescens*		*P. sylvestris*		Mean
	Tree 42-1	Tree 50-1	Tree C-12	Tree 48-2	Tree V-15	Tree V-16	Tree R596	Tree S493	
1. Check[b]	40	87	84	95	93	98	100	99	85
2. *Rhizoctonia solani*	54*	83	81	86	50*	46*	74*	72*	68*
3. *Panagrolaimus* sp.	44	84	87	86	92	90	99	100	85
4. *Alternaria* (*tenuis*?)	42	94	77	95	87	98	93	96	85
5. Mixture of 2, 3, 4, 6, and 7	42	81	89	91	92	98	95	97	86
6. *Pythium* (*debaryanum* ?)	40	82	77	97	94	96	100	99	86
7. *Fusarium oxysporum* var. *redolens*	52	85	87	92	97	98	94	97	88

[a] Asterisk indicates that the difference between the indicated figure and the corresponding one for the check is statistically significant at the 5 per cent level.
[b] Soil treated with Tersan.

potentials for caragana were established with *P. debaryanum* and *P. ultimum* (8 isolates), *C. praticola* (6 isolates), *F. solani* (4 isolates), *F. acuminatum* (16 isolates), *F. oxysporum* (6 isolates), and *Alternaria* spp. (10 isolates). The degree of pathogenicity of these fungi varied in the above order.

The data of Tables 1 and 2 demonstrate the relative virulence of damping-off isolates as well as their pathogenic potential on young seedlings, i.e. their ability to kill under conditions favoring pathogenicity.

INOCULATION IN NONSTERILIZED SOIL.—*Pre-emergent damping-off.*—The germination, recorded as the emergence percentage, is shown in Table 3. A statistically significant difference in germination between treatments and check indicates pre-emergent damping-off.

Reisolation attempts from nongerminated seeds, after the conclusion of the experiment, failed because the parasites were already replaced by other fungi. platings of nongerminated seeds often were sterile (28 per cent) or gave fungi considered as saprophytes. A number of the seeds were alive but dormant; 26 per cent of the nongerminated seed was classified as "sound."

The germination of the seed of the white spruce tree 42-1 was increased by Rhizoctonia and by Fusarium. The increase was statistically significant with the former and almost so with the latter. Possible explanations are that these fungi reduced seed dormancy by a disintegrating action on the impervious seedcoats or that they stimulated germination by means of excretions. *R. solani* has been found to stimulate the growth of various plants that are fairly resistant to damping-off (24).

Postemergent damping-off.—The percentages of seedlings that died are shown in Table 4. In tests with caragana, losses in the check treatment and in most other treatments were negligible. Losses in Rhizoctonia treatment were twice those in the Pythium treatment and 6 times those in the check treatment. Statistically significant differences among tree species and among the progenies of different trees existed. Losses for tree 42-1 were generally larger than those for the other white spruce, 50-1. In contrast, seedling losses for tree 42-1 were relatively small in the Rhizoctonia treatment. The germination of this progeny actually was increased by Rhizoctonia (Table 3).

Seedling losses for tree C-12 were consistently less than those for the other Colorado spruce tree; the differences were statistically significant for Fusarium and Rhizoctonia treatments. These cases suggest a genetical difference for resistance to damping-off.

Reisolations.—Several fungi were isolated from each seedling in most instances. In many cases, no fungi (14 per cent) or only bacteria, were obtained. Patho-

TABLE 4.—*Percentage losses*[a] *of seedlings from 2 trees each of Picea glauca, P. pungens, Pinus sylvestris, and Caragana arborescens in tests with nonsterilized soil*

Inoculum	*P. glauca*		*P. sylvestris*		*P. pungens*		*C. arborescens*[b]		Mean
	Tree 42-1	Tree 50-1	Tree R596	Tree S493	Tree 48-2	Tree C-12	Tree V-15	Tree V-16	
1. Check[c]	(5)	(7)	(6)	(5)	(14)	(7)	(1)	(0)	(7)
2. *Fusarium oxysporum* var. *redolens*	13	24	9	7	23*	7	(0)	(1)	14
3. *Alternaria* (*tenuis* ?)	18	21	14	10	20	19	(0)	(0)	17
4. *Panagrolaimus* sp.	27	20	11	21	17	10	(0)	(0)	18
5. *Pythium* (*debaryanum* ?)	43*	9	27	16	15	13	(0)	(0)	21
6. Mixture of 2, 3, 4, 5, and 7	65*	12	13	15	19	7	(1)	(1)	22
7. *Rhizoctonia solani*	30	40*	65*	62*	45*	23	(72)	(65)	44*

[a] Asterisk indicates that the difference between the indicated figure and the corresponding one for the check is statistically significant at the 5 per cent level.
[b] Data for *C. arborescens* not included in analyses but given for comparison.
[c] Data for check (Tersan-treated soil) for comparison only.

gens were followed by secondary invaders from the natural soil flora, immediately after or perhaps even before disease symptoms appeared (Table 5). The pathogens could have been inhibited by some of these secondary invaders and by surface contaminants (30). In some cases the pathogens probably were killed when decayed tissues imbibed too much surface sterilizer; therefore a very high incidence of reisolation was not possible. The isolates under the heading "other fungi" in Table 5 consisted mainly of species of *Trichoderma, Cephalosporium, Gliocladium, Penicillium, Aspergillus, Stemphylium, Mucor, Phoma,* and *Streptomyces.* These are generally considered as saprophytes or pathogens of low virulence.

In the Rhizoctonia treatment, the reisolation incidence was high for all trees, varying from 21 to 61 per cent. For tree 42-1 it was 23 per cent. The reisolation of Pythium for this tree was 50 per cent in the Pythium treatment and 33 per cent in the mixture treatment. In the latter, no Rhizoctonia was reisolated and Fusarium incidence was 67 per cent. For tree 48-2 in the Fusarium treatment, the Fusarium incidence was 48 per cent and the Pythium incidence was 33 per cent.

Pathogenicity of the inocula.—Rhizoctonia was considered pathogenic to seeds of pine and caragana despite the failure in reisolations from seeds. Pathogenicity was indicated by the significant reductions in germination following the Rhizoctonia treatment (Table 3). This fungus was considered highly pathogenic to seedlings of pine, caragana, and Colorado spruce, and of 1 tree (50-1) of white spruce (Table 4). This conclusion is supported by the frequent reisolation of Rhizoctonia from diseased seedlings in the Rhizoctonia treatments but not in other treatments nor in any surviving seedlings (Table 5).

TABLE 5.—*Incidence of various fungi among isolates recovered from inoculated seedlings in tests with nonsterilized soil*

Original inoculum	Percentage incidence of isolates: Rhizoctonia	Pythium	Fusarium[a]	Other fungi
From dying seedlings				
1. Check[b]	0	9	61	26
2. *Fusarium oxysporum* var. *redolens*	0	25	53	15
3. *Alternaria* (*tenuis* ?)[c]	0	24	27	34
4. *Panagrolaimus* sp.[c]	0	22	33	37
5. *Pythium* (*debaryanum* ?)	0	14	43	30
6. Mixture of 2, 3, 4, 5, and 7	11	20	47	14
7. *Rhizoctonia solani*	48	6	14	14
From surviving seedlings				
1. Check[b]	0	2	10	2
2. *Fusarium oxysporum* var. *redolens*	0	0	68	13
3. *Alternaria* (*tenuis* ?)[c]	0	0	12	80
4. *Panagrolaimus* sp.[c]	0	2	44	66
5. *Pythium* (*debaryanum* ?)	0	0	43	35
6. Mixture of 2, 3, 4, 5, and 7	0	0	88	21
7. *Rhizoctonia solani*	0	0	23	77

[a] Mostly *Fusarium oxysporum* but not verified to be the inoculum strain.

[b] Soil was treated with Tersan.

[c] Reisolation incidence of Alternaria was 7 per cent and that of the nematode was 3 per cent; these also occasionally were recovered from seedlings (both dying and surviving) that received other inocula.

No significant pathogenicity was accredited to the Alternaria and nematode inocula because of the very low reisolation of these and the relatively low losses in the treatments. Conclusions on the pathogenicity of the other inocula were complicated by the fact that natural Fusarium and Pythium inocula were obviously present in the experimental soil (Table 5).

With the exception of caragana, seedling losses in checks were consistently less than those in any of the other treatments. Where Rhizoctonia was absent, the losses probably were caused by Pythium, by Fusarium, or by both. The main pathogen was Pythium, for the progeny of tree 42-1 at least, as this inoculum was associated with high losses and Pythium was reisolated frequently. Resistance of this progeny to Rhizoctonia is indicated by the data in Tables 3 and 4. In addition, isolation data suggest that in the mixture treatment most seedling mortalities of this progeny were caused by Pythium rather than by Rhizoctonia.

The significantly high losses in Fusarium treatment for the progeny of tree 48-2 (Table 4) with frequent reisolation suggest that this inoculum was somewhat pathogenic. Nevertheless, part of the losses probably were caused by the natural Pythium inoculum as suggested by the isolation data. Fusaria frequently were isolated even from apparently healthy seedlings (Table 5), so that their generally high incidence cannot be considered to invalidate the conclusions of the pathogenicity of Rhizoctonia or of Pythium. Possibly Fusaria occurred mostly in dead surface cells and root hairs and were capable of restricted or slow pathogenic action only. In sterilized medium in test tube experiments, Fusarium inoculum similarly was less virulent than Rhizoctonia or than Pythium (Tables 1, 2).

All inocula appeared less pathogenic in the presence of soil flora (Tables 3, 4) than in their absence (Tables 1, 2). The differences between the treatments of Rhizoctonia and the mixture in Tables 3–5 suggest that some of the inocula were strongly antagonistic to Rhizoctonia.

DISCUSSION.—In addition to establishing the pathogenicity of certain fungi to seed and seedlings of certain trees, some points of general interest were revealed by this study. The various gradations from high to low pathogenic potentials, as exhibited by the fungi listed in Tables 1 and 2, recently have been found in similar pathogenicity studies on red clover in Wisconsin (13). The latter work involved many fungi related to those listed in Tables 1 and 2, and the results of the 2 investigations were fairly similar, e.g.,

with species of *Fusarium*, *Gliocladium*, *Phoma*, *Trichoderma*, *Alternaria*, and *Aspergillus*.

Frequent isolations of Fusaria from apparently healthy plants (Table 5) also have been recorded in studies with Sitka spruce (31) and citrus roots (25). This, contrasted with the absence of *R. solani* (Table 5) (23) and Pythium (Table 5) in healthy tissues, suggests that mere isolations of Fusaria are doubtful indications of virulent pathogenicity; however, many Fusaria may be capable of weakly pathogenic action, such as slow root rotting of trees. They are known to cause root rot of clover and alfalfa (5). Tint's (27) inoculation studies under sterile conditions suggest that certain strains of *Fusarium* may be much more virulent on coniferous seedlings than are most of those tested in the present work.

Pythium was very virulent in the tests reported in Table 1 but not so in the tests with nonsterile soil reported in Tables 3 and 4. Two factors may have caused this. First, the cautious though frequent irrigation of the soil may have been insufficient for heavy infection. Maximum mortality by certain species of Pythium, in contrast to *R. solani*, requires saturated soil (1, 18, 19, 31). The frequent pure isolation of Pythium in certain epidemics in nurseries during heavy rains suggests high potential virulence. Second, antibiosis probably influenced Pythium in the experimental soil. The suppression of Pythium by bacteria from the experimental soil was actually found in petri dish experiments (30). The suppression of Pythium by certain nonsterilized nursery soils also has been demonstrated in studies with pine species by Hartley (11), with Sitka spruce by Warcup (31), and with 3 broadleaved species by Wright (35). The hypothesis that antibiosis is important in forest nurseries previously has been given by Büttner in 1903 (3). Hartley (11) confirmed this by pot experiments in which Pythium caused significantly more damping-off when alone than when mixed with 11 saprophytes.

Studies with agricultural crops have shown the importance of natural antibiotic control of Pythium damping-off (10, 15, 21, 22). Bacteria, actinomycetes, or certain fungi have appeared as the main antagonists. The natural antibiotic control of damping-off, however, varies with soil (10, 14, 15, 20, 24, 26, 28, 31), with previous crops (9, 23), with temperature (10), and with other factors (10). An attempt to predict damping-off incidence from details of soil flora has failed (36).

Rhizoctonia is often very virulent even in nonsterile nursery soil (19, 20, 21, 23, 24, 26, 33, 35) (see also Tables 3, 4). Some experiments have shown synergism rather than antagonism with soil flora (20, 22, 24, 35). On the contrary, many common fungi are known as antagonists or even parasites of Rhizoctonia (10, 14, 26, 32, 33). *Trichoderma viride* and species of *Gliocladium*, *Penicillium*, *Cylindrocarpon*, and *Streptomyces*, amongst others, are reported to antagonize *R. solani*. These occurred in the experimental soil but did not greatly decrease the virulence of Rhizoctonia. Evidently the mere presence of these antagonists usually is not sufficient to control Rhizoctonia. The same conclusion has been suggested by other investigators (9, 21, 31, 33, 36). In contrast, mixing Rhizoctonia inoculum with other inocula greatly reduced its virulence (Tables 3, 4). Explanations of this are strong competition or the action of Panagrolaimus with the associated antagonistic bacteria. A possibility is that a complex change in microbiological balance occurred; extremely complex relations among soil fungi have been experimentally demonstrated (14).

Antagonists can also affect Fusarium (24, 26) and cause otherwise fast-growing species of *Fusarium* to grow very slowly in soil (16). Analogously, the Fusarium inoculum, which was moderately virulent in test tubes (Tables 1, 2), caused little or no damping-off in the soil tests (Tables 3, 4).

The apparent nonpathogenicity of Alternaria (Tables 3, 4) also may be due to antibiosis. This hypothesis is supported by the fact that in isolations in the field, this fungus usually was confined to the top of seedlings that were invaded by other organisms from soil. Alternaria may be incidental as a damping-off associate, but the possibility cannot be excluded that it is a weak pathogen of trees (37) under certain conditions, primarily on leaves or needles.

FOREST PATHOLOGY LABORATORY
SCIENCE SERVICE, DEPARTMENT OF AGRICULTURE
SASKATOON, SASKATCHEWAN
AND
FOREST NURSERY STATION
EXPERIMENTAL FARMS SERVICE
DEPARTMENT OF AGRICULTURE
INDIAN HEAD, SASKATCHEWAN

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CERCOSPORA BLACK STEM OF ALFALFA [1]

J. W. Baxter

SUMMARY

Cercospora black stem is an important disease of alfalfa in Iowa. The leaf spot phase of the disease appears in mid-June, followed by stem discoloration during late summer and early autumn. The causal fungus, *Cercospora medicaginis* Ell. & Ev., overwinters chiefly as mycelium in plant debris. The incidence of seed infection in alfalfa appears to be low.

In laboratory studies of *C. medicaginis*, the optimum temperature for both growth in culture and germination of conidia was found to be near 25°C. The fungus failed to sporulate on acidified potato-dextrose agar, lima bean agar, malt agar, carrot agar, or cornmeal agar. Sporulation occurred on carrot leaf agar, on sterile stems of alfalfa, red clover, and sweetclover, and in carrot decoction. Abundant sporulation also was induced by flooding plates and slants of nonacidified potato-dextrose agar with mycelial suspensions of 2- to 4-week-old isolates and by growing the fungus in shake cultures, using a mixture of Richard's solution and malt extract as the culture medium.

In greenhouse inoculations with conidia of *C. medicaginis*, the fungus proved to be pathogenic to species of Medicago but failed to infect species of *Melilotus* and *Trifolium*. Penetration of the host by germinating conidia was observed 48 hours after inoculation. Entrance was effected through stomata, without the formation of appressoria. There was no evidence of direct penetration.

The black stem complex is an important disease of alfalfa, red clover, and sweetclover in north central United States. On alfalfa, at least 2 organisms have been found to be associated with black stem. These are *Ascochyta imperfecta* Pk., which appears to be most abundant during the cooler weather of spring and late autumn, and *Cercospora medicaginis* Ell. & Ev., which causes stem blackening and leaf spotting during summer and early autumn. Disease surveys made by the writer in Iowa during 1953, 1954, and 1955 indicate that *C. medicaginis* is of considerable importance as an alfalfa pathogen. This paper reports the results of a study of Cercospora leaf spot and black stem of alfalfa, particularly with respect to the physiology, host range and life history of the causal fungus.

THE DISEASE.—Under Iowa conditions the disease first appears in mid-June, as small brown spots on the leaves. These spots enlarge to form circular lesions, reddish brown or smoky brown in color and from 2 to 6 mm in diameter (Fig. 1). When environmental conditions favor sporulation, the lesions become ashy gray in color because of the abundant production of conidia. In heavy infections, entire leaflets are killed and severe defoliation occurs. The leaf spot phase is followed by the appearance of dark brown elliptical or linear lesions on petioles and stems. As the season progresses these lesions enlarge and coalesce. Under favorable conditions for disease development, entire stems become discolored. Smaller stems, petioles, and peduncles may be killed, resulting in further defoliation and loss of seed.

Leaf lesions caused by *C. medicaginis* show some resemblance to those of Stemphylium leaf spot, caused by *Stemphylium botryosum* Wallr. Lesions of Stemphylium leaf spot, however, can be distinguished by their elliptical or irregular shape and by the frequent presence of concentric rings. The stem discoloration caused by *C. medicaginis* on alfalfa is similar in appearance to that caused by *Ascochyta imperfecta* but is lighter in color. Stem lesions due to infection by *A. imperfecta* are black to blackish brown, whereas those caused by *C. medicaginis* range from reddish brown to chocolate brown.

THE CAUSAL ORGANISM.—On the basis of a morphological study of *Cercospora medicaginis* on Medicago, *C. davisii* on Melilotus and *C. zebrina* on Trifolium,

FIG. 1. Leaf spot of alfalfa caused by *Cercospora medicaginis*.

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Horsfall (3) reduced the first 2 species to synonymy with *C. zebrina*. The results of cross-inoculation experiments by Nagel (5) and Jones (4) indicate that these 3 species of *Cercospora* are specialized at the host genus level. In a recent monograph of the genus *Cercospora*, Chupp (1) treats *C. medicaginis*, *C. davisii*, and *C. zebrina* as distinct species.

The isolates of *C. medicaginis* used in the present study agreed closely in morphology with published descriptions of the fungus. Conidiophores and conidia showed considerable variability in length and number of septations. In moist chambers and on sterilized stems in test tubes, conidiophores and conidia were much longer than were those produced in drier environments. Such environmental differences also were expressed in the size of the stromata. Stromata formed in culture and in moist chambers were larger than those produced under natural conditions.

HOST RANGE.—The susceptibility of 6 species of legumes in 3 genera was determined in greenhouse inoculations in which suspensions of conidia were used as the inoculum. Tests were made on the following species: *Medicago sativa* L., *M. lupulina* L., *M. hispida* Gaertn., *Melilotus officinalis* (L.) Lam., *M. alba* Desr., and *Trifolium pratense* L. In 3 experiments only the 3 species of *Medicago* proved to be susceptible.

SPORULATION IN CULTURE.—Hopkins (2) reported stimulation of conidial production in *C. medicaginis* by partial drying of the agar in plate cultures of the fungus. Nagel (6) induced sporulation in *C. davisii* by growing fresh conidial isolates on potato-dextrose agar within a temperature range of 23°–27°C. He was able to maintain his cultures in a sporulating condition by transferring at intervals of 3 or 4 days.

In the present study, 6 isolates of *C. medicaginis* were grown on a wide variety of culture media in an attempt to induce sporulation. Under ordinary conditions, all 6 isolates failed to sporulate on potato-dextrose agar, lima bean agar, malt agar, carrot agar, cornmeal agar, and prune agar. Sparse sporulation occurred on prune agar when cultures were allowed to dry and were then flooded with sterile distilled water. More abundant production of conidia occurred on carrot-leaf agar, on alfalfa-leaf agar, and on stems of alfalfa, red clover, and sweetclover that had been autoclaved for 15 minutes at 12 lb. pressure; however, the sporulation induced by these media was not sufficiently abundant to supply the amount of inoculum necessary for screening large numbers of plants for resistance.

In a further investigation of the problem, production of morphologically abnormal but viable conidia was stimulated by growing mycelial suspensions of *C. medicaginis* in shake cultures, using a mixture of equal parts of Richards' solution and malt extract as the culture medium. This method, however, did not give consistent results. In further work on the induction of sporulation, 2 other methods were tested. The first consisted of flooding plates and slants of non-

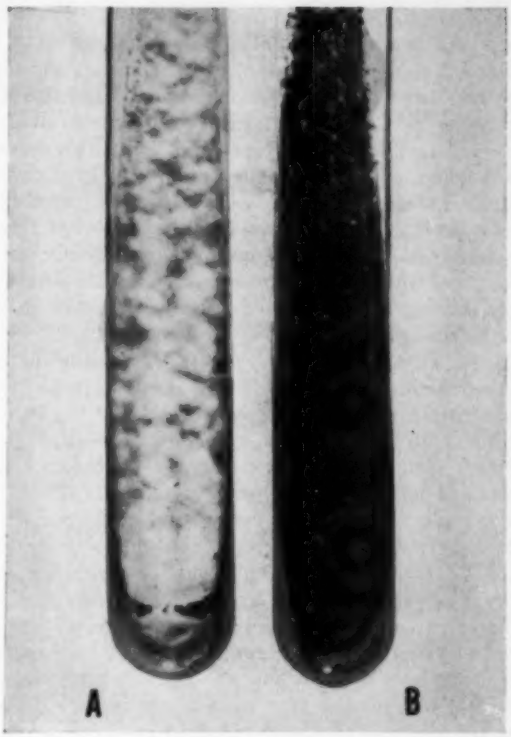

FIG. 2. Cultures obtained by flooding potato-dextrose agar slants with mycelial suspensions of *C. medicaginis*. A) Nonsporulating culture produced after flooding with a suspension of a 14-month-old isolate. B) Sporulating culture produced after flooding with a suspension of a fresh isolate.

acidified potato-dextrose agar with mycelial suspensions of 2- to 4-week-old isolates of the fungus. This resulted in abundant production of conidia. The sporulating cultures thus obtained had a dark appearance due to the superficial formation of dark-colored stromata and conidiophores. On plates and slants that had been flooded with mycelial suspensions of 14-month-old isolates, only white fluffy aerial mycelium was produced on the colony surface (Fig. 2, A). In addition to this technique, the carrot-decoction medium used by Sherwin and Kreitlow (7) was tested to determine its effect on *C. medicaginis*. This medium stimulated sporulation in both old and fresh isolates of the fungus.

EFFECT OF TEMPERATURE ON GROWTH AND SPORE GERMINATION.—The effect of temperature on the rate of growth in culture was determined. Tests were made at 5° intervals from 5° to 35°C. Each culture used in this test was grown on potato-dextrose agar and was started from a 4-mm disk of mycelium. Measurements of colony diameter were made at the end of a 21-day period. Good growth was obtained at 20°, 25°, and 30°C, with the optimum at 25°.

Further data on the effect of temperature on the organism were obtained by germinating conidia in sterilized distilled water on glass slides at 5° intervals

over a temperature range from 5° to 35°C. The slides were kept in Petri dish moist chambers and were examined 2, 3, 6, and 12 hours after beginning the tests. The results of 4 tests indicated an optimum temperature near 25°C. The manner of germination also was observed in these tests. Growth of germ tubes began 2.5–3 hours after the conidia were placed in the water drops. In most conidia, germination occurred first in the basal cell and next in the terminal cell, followed by germination of several intercalary cells. Occasionally, 2 germ tubes were produced from a single cell of a conidium; this occurred most frequently in the basal cell.

Host penetration.—A study was made of the method of host penetration by *C. medicaginis*. Pieces of inoculated leaves were removed 6, 12, 24, 48, and 72 hours after inoculation, cleared in acetic acid and alcohol, stained with cotton blue in lactophenol, and examined for evidence of penetrating germ tubes. Although many conidia had germinated at the end of the 6-hour period, no evidence of penetration was observed until 48 hours after inoculation. Considerable difficulty was experienced in finding germ tubes that had penetrated; an examination of more than 400 conidia revealed only 3 with germ tubes that had entered the host plant. Penetration was accomplished through stomata, without the formation of appressoria. There was no evidence of direct penetration.

Overwintering.—Infected stems of alfalfa that had overwintered in the field were brought into the laboratory on March 3, washed in running tap water, dipped in alcohol for 30 seconds, and placed in moist chambers. Conidiophores and conidia of *C. medicaginis* were produced on these stems 7–10 days later. Isolates were obtained by removing stromata or clumps of conidiophores from the substrate with a sterile needle. These isolates showed cultural and morphological characteristics typical of *C. medicaginis* and proved to be pathogenic on alfalfa.

To determine whether or not the fungus is seedborne, 100 seeds from each of 65 samples of alfalfa seed were surface sterilized and plated out on potato-dextrose agar. Isolates of *C. medicaginis* were obtained from 11 seeds representing 6 samples. In further studies on seed infection, 5 samples of seed were harvested from 2 plots of Du Puits alfalfa known to be severely affected by Cercospora leaf spot and black stem. One hundred seeds from each sample were surface sterilized by the method employed by Hopkins (2) and then plated out on potato-dextrose agar. No isolates of the fungus were obtained from these seed samples.

Department of Botany and Plant Pathology
Iowa State College
Ames, Iowa

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LIGHT AND ELECTRON MICROSCOPY OF X-BODIES ASSOCIATED WITH BROAD-BEAN MOTTLE VIRUS AND PHASEOLUS VIRUS 2[1]

M. Rubio and D. H. M. van Slogteren

SUMMARY

X-bodies associated with broad-bean mottle virus in the cells of *Vicia faba* were found to be usually ovoid in form. They appeared granular under the light microscope, became vacuolate after 2 to 3 weeks, and were composed entirely or mainly of spherical virus particles that were visible in electron micrographs.

Under the light microscope, the X-bodies associated with Phaseolus virus 2 appeared similar to those of broad-bean mottle virus. Under the electron microscope, they were seen to be composed mainly of amorphous material. A few aggregated flexuous rod-shaped virus particles were present, but it was not determined whether these were in or were adsorbed to the surface of the X-bodies.

The broad-bean virus X-bodies were found only in the cells in chlorotic areas of the leaf, whereas the Phaseolus virus 2 inclusions were found in all parts of the leaf and stem.

Some properties of a new virus in legumes, broad-bean mottle virus, have been described by Bawden, Chaudhuri, and Kassanis.[2] Working with broad beans infected with this virus, we found characteristic granular X-bodies. Since these bodies resembled those described by McWhorter[3] in plants infected with Phaseolus virus 2, it seemed desirable to study each by means of the light and electron microscopes.

MATERIALS AND METHODS.—We are indebted to Mr. F. C. Bawden, of Rothamsted Experiment Station, for kindly forwarding a sample of broad-bean mottle virus to our laboratory. The Phaseolus virus 2 strain used was obtained by inoculating broad beans with juice of infected gladiolus plants. Broad bean (*Vicia faba* L.) was used as a host for both viruses. Infected plants were studied in comparison to healthy plants.

Strips of epidermal tissue were stained for X-bodies with a 0.5 per cent solution of phloxine in distilled water as described by Rubio[4]. Some observations were carried out on strips stained with trypan blue according to the method of McWhorter[5] and others on strips without stain. Preparations for permanent use were fixed with Carnoy fluid.

Preparations for the electron microscope were made of phloxine-stained epidermal strips. These were not fixed, but they were thoroughly washed with tap water followed by distilled water and then shredded with dissecting needles to allow the cellular contents to come into the water. Because epidermal cells are practically devoid of chloroplasts, only the X-bodies, the host nuclei, and some cell wall remains were detectable in the distilled water.

Under the light microscope, at low magnification, the stained X-bodies were easily recognized and could be picked up with a micropipette and mounted on the grid of the electron microscope. The grids were then dried, shadowcast with palladium, and examined in an electron microscope. We are much indebted to Mr. J. D. Bakker for making the electron micrographs.

RESULTS.—X-bodies of broad-bean mottle virus in *V. faba* were found only in the chlorotic areas of infected leaves. Our observations indicated that the X-bodies are granular during the first and second stages of their formation; they later become vacuolate. Their size varied according to the stage of formation, from small granules to bodies several times the size of the nuclei (Fig. 1, A).

The X-bodies apparently were formed from granules in the way described by Sheffield[6] for aucuba mosaic virus. They withstood desiccation and were not greatly damaged when touched with a needle. They were deeply stained with phloxine and less easily with trypan blue.

Preparations for electron microscopy showed that the X-bodies are composed mainly or entirely of spherical particles (Fig. 1, B) that both in size and shape are apparently identical to the virus particles of broad-bean mottle virus as described by Bawden et al[2] in purified preparations.

McWhorter[3] has described ovoid and crystalline inclusions of Phaseolus virus 2. To date we have been able to find only ovoid inclusions (Fig. 1, C) in our work on Phaseolus virus 2 in *V. faba*. It is of interest to note that whereas the X-bodies did not differ from those of broad-bean mottle virus in gross appearance, their distribution in the leaf tissue was quite different. In *V. faba* infected with Phaseolus virus 2, X-bodies were found in almost every cell of the epidermis of

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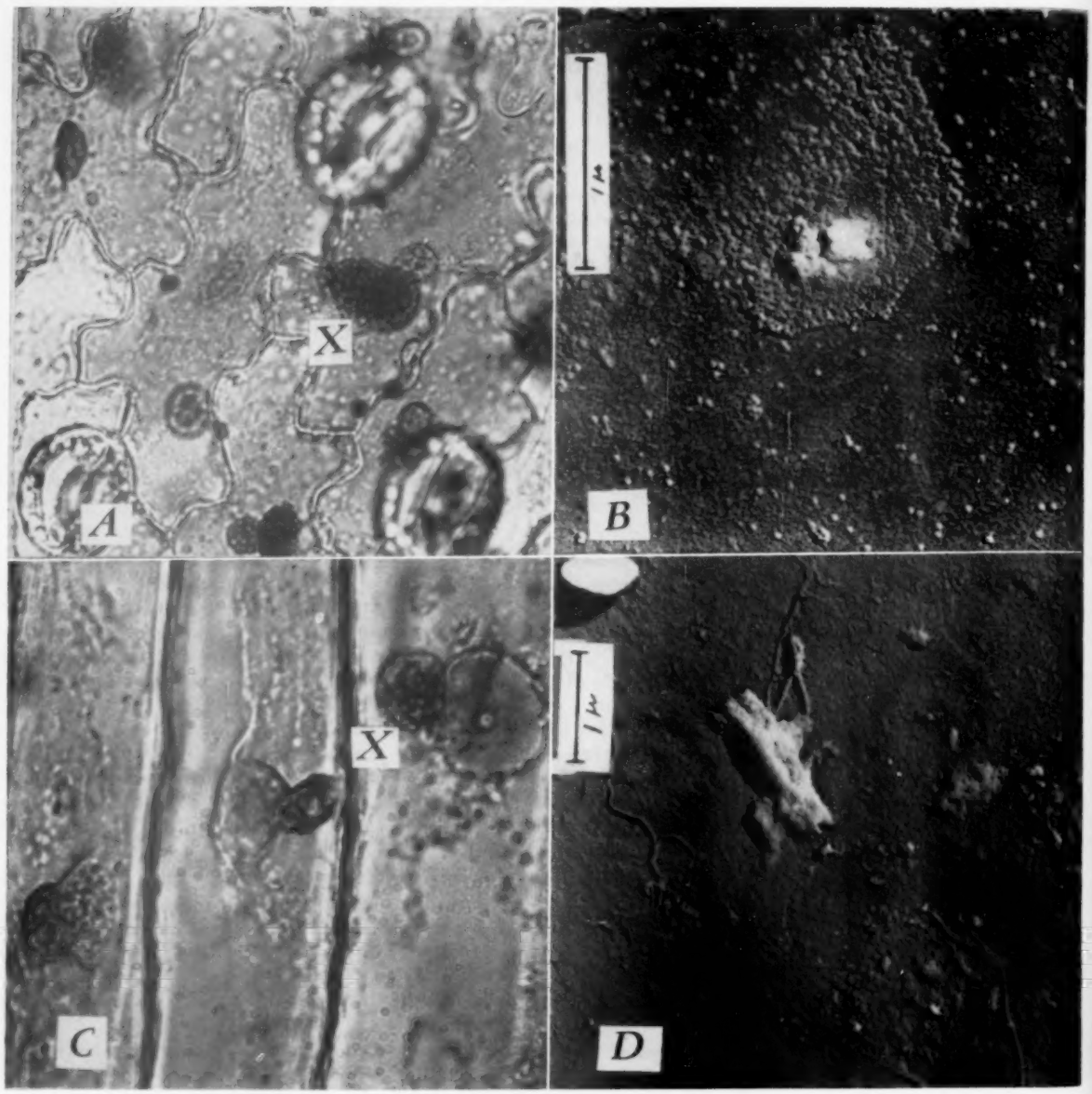

FIG. 1. Virus inclusion bodies in bean. A) Light photomicrograph of epidermal cells showing inclusion (X) associated with infection by broad-bean mottle virus; B) Electron micrograph of disintegrating cell inclusion of broad-bean mottle virus showing that the inclusion is made up mainly of spherical virus particles. C) Light photomicrograph of epidermal cells showing inclusion body (X) associated with infection by Phaseolus virus 2. D) Electron micrograph of disintegrated inclusion body of Phaseolus virus 2. Note the aggregated flexuous rods. Their exact relation to the inclusion body is not known.

the leaves and the stem, whereas in plants infected with broad-bean mottle virus, the bodies were found only in the chlorotic areas in the leaves and none were found in the stem.

Preparations of X-bodies of Phaseolus virus 2 for electron microscopy were made in the same way as described herein for those of broad-bean mottle virus. The electron micrographs (Fig. 1, D) indicate that most of the X-body is composed of more or less amorphous material, with a few aggregated flexuous virus rods also present. These rods resemble those in purified virus preparations from infected plants. It was not determined whether the rods observed were in the X-bodies or adsorbed to their surface.

INSTITUTO DE EDAFOLOGIA, MADRID, SPAIN
AND
LABORATORIUM VOOR BLOEMBOLLENONDERZOEK,
LISSE, HOLLAND

SPHACELOTHECA CRUENTA. 2. GROWTH CHARACTERISTICS IN SYNTHETIC MEDIA AND OXIDATIVE METABOLISM[1]

Cyril W. Blackmon[2] and Roy B. Mefferd, Jr.[3]

SUMMARY

Sphacelotheca cruenta grew well in a simple inorganic medium (Czapek's) with sucrose as the sole carbon source over a wide range in pH (4.5–8.0) and at temperatures from 5 to 30°C. No growth occurred at pH 3 or pH 9, or at 37°C. Many organic compounds were oxidized and supported growth. Generally, compounds that supported growth also were oxidized. Exceptions to this were the polysaccharides of which only dextrin was oxidized. Very few differences were noted between the ability of cells in the mycelial stage and those in the sporidial (yeast-like) stage to oxidize the various substrates, except that generally the latter oxidized the various substrates tested more rapidly. The sporidial cultures utilized dextrin and maltose much less readily than did mycelial cultures. Apparently, dextrinase and maltase activities were low in sporidial cells, possibly due to their having been grown on a richer medium than the mycelial cells. Nitrogen-containing compounds were utilized more slowly than the carbohydrates.

For an understanding of the nature of the host-parasite relationship and of specificity, knowledge of the pathological process must be coupled with intimate knowledge of the nature of the parasitic organism—of its cytological, genetic, and growth characteristics and of its enzyme systems. The physiology and metabolism of very few of the Basidiomycetes have been studied in detail (3, 7). *Sphacelotheca cruenta* (Kühn) Potter is virtually uninvestigated in this respect. Since *S. cruenta* can be grown on artificial media in pure cultures (2) and has a relatively low degree of host specificity (6), it is well suited for the subject of an investigation into the biochemical roots of host specificity.

On rich media, *S. cruenta* tends to grow as a yeast-like (sporidial) form rather than as a mycelial culture. This would lend credence to the idea that the nutritional requirements of the various types of cultural growth forms, of primary and secondary mycelia, or even of different mating types may vary. These various forms may have somewhat different enzymatic systems, or the enzymes for one reason or another may have different activities. Considerable differences in physiological behavior have been noted between monocaryotic and dicaryotic organisms of the same species (1, 5).

This report covers determination of optimal pH, temperature and carbon source for *S. cruenta* growing in pure culture on a synthetic medium containing only 1 organic compound, and an exploratory survey of the relative abilities of the intact cells of mycelial and yeast-like cultures of *S. cruenta* to oxidize a group of organic compounds.

MATERIALS AND METHODS.—*Cultures for manometric experiments.*—A seemingly typical single colony of *S. cruenta* growing in solid Czapek's medium was picked and carried in stock culture with repeated transfers of single colonies to insure stabilization of the culture. When mycelial growth was desired, 3-liter Erlenmeyer flasks containing 1 liter of sterile Czapek's broth enriched with 0.5 per cent Difco nutrient broth were seeded from the stock culture. Sporidial or yeast-like growth was obtained by seeding Czapek's liquid medium enriched with 2 per cent Difco nutrient broth with a subculture of the stock culture that was growing in the sporidial form. Czapek's medium was supplemented in both cases so as to obtain rapid and heavy growth. The differential in the amount of supplement used, though undesirable, was necessary to insure that the cultures grew in a mycelial or yeast-like form and that either produced adequate growth within the same time intervals.

The cultures were incubated on a mechanical shaker at 24°C until a standard turbidity was attained, which occurred usually in 3 days and always in 4. The turbidity selected was such that rapidly growing young cultures uniformly resulted. The mycelia grew dispersed through the medium, with practically no sporidia being formed under these conditions. The sporidial cultures resembled yeast cultures with no mycelia.

The cultures were placed into sterile Waring Blendor jars and stirred for 1 minute, with the motor speed reduced to ¼ normal by the aid of a rheostat. This process broke the mycelial suspension into small fragments several mm in length. The resulting suspension could be pipetted uniformly into Warburg flasks. The sporidial cultures were similarly treated. The cells were then centrifuged and washed 5 times in sterile 0.02 M phosphate buffer at pH 6.5. After the last washing, the cell suspensions were diluted to a standardized turbidity such that 2 ml had an approximate

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From a thesis submitted by the senior author in partial fulfillment of the requirements for a M.S. degree in the Graduate School of Trinity University. Appreciation is expresed to Mrs. Wilma Jean Blackmon for technical assistance and to Dr. F. W. Bieberdorf for suggesting the problem.

[2] Present address: Department of Horticulture, Northeast Louisana State College, Monroe, Louisana.

[3] Present address: Biochemical Institute, University of Texas, Austin, Texas.

dry weight of 30 mg. The actual dry weights were determined each time by drying at 100°C. to a constant weight.

After the washing in phosphate buffer, all cultures were incubated at 70°C for 12 hours, then used within the next 12 hours. Earlier experiments showed that variable results were obtained in cultures allowed to incubate for longer periods—the enzymatic activity dropped steadily after 24 hours until the fourth or fifth day, when no respiration on the various substrates was observed. Just prior to use, yeast extract (Difco) was added to the cultures at the rate of 1 ml of a 0.01 per cent solution per liter of cell suspension. The extensive washing and the incubation in the absence of added substrate were essential to decrease the otherwise very high endogenous rate of respiration. Otherwise, this rate was so great that it practically precluded stimulation of respiration by the addition of any of the substrates.

Manometric procedures.—Oxygen uptake was measured by standard manometric techniques at 26°C in air (11). After 20 minutes' equilibration, 1 ml of a 0.02 M solution of substrate dissolved in 0.02 M phosphate buffer at pH 6.5 was added from the sidearm of 2 ml of the cell suspension (dry weight ranged from 28.0 mg to 33.8 mg in the various experiments). The center well contained 0.2 ml of 20 per cent KOH and a 2.5-cm-square accordion of quantitative filter paper. Flasks were run in duplicate, and the oxygen uptake for each substrate was determined at least once more with a different culture.

Substrates were sterilized by filtration except for the polysaccharides, which were autoclaved double strength in distilled water, then added to sterile double-strength phosphate buffer. Each flask was examined at the conclusion of the experiment (2–4 hours) for evidence of gross contamination. The results were discarded if such was found. The duplicate values were averaged for each substrate, and the QO_2 values (dry-weight basis) were determined.

Growth studies.—Czapek's medium, slightly modified, was employed throughout and was prepared as follows: $NaNO_3$, 3 g/l; K_2HPO_4, 1 g/l; KCl, 0.5 g/l; $MgSO_4 \cdot 7H_2O$, 1.5 g/l; $FeSO_4 \cdot 7H_2O$, 0.005 g/l; sucrose, 20 g/l. Where carbon sources other than sucrose were employed, the compound was substituted for sucrose at the same concentration. This concentration exceeded the solubilities of a number of the compounds, e.g. starch, cystine, cysteine, and tyrosine; therefore a nonseeded tube was used in every series as a turbidity blank. All media were sterilized by filtration through bacteriological Seitz filters, except for the polysaccharides and those compounds that did not dissolve completely. These were autoclaved in distilled water. Media were autoclaved only where it was necessary in order to avoid possible degradation of the organic constituents (10).

Cells picked from a single colony on Czapek's solid medium (1.5 per cent agar) were seeded into liquid Czapek's medium and grown for 5 days with some agitation. Yeast-like growth resulted. These cells were diluted with phosphate buffer (0.02M, pH 6.5) so as to contain from 10,000–20,000 cells per ml. The parent culture had been allowed to stabilize by being repeatedly grown on Czapek's agar for a period of 6 months.

In tests on optimal pH for maximum growth, different lots of Czapek's media were adjusted with phosphate to various pH levels that differed by 0.5 pH from pH 3.0 to 9.0. Five ml each of these media was pipetted aseptically into each of 3 sterile test tubes (27 × 80 mm) and seeded with 0.5 ml of standardized inoculum. The tubes were placed on a reciprocating shaker with a stroke of 4.8 cm. The speed was adjusted so as to agitate gently the broth in tubes inclined at a 45° angle. Tubes were tilted to increase the surface area exposed to air, thus preventing oxygen from becoming a limiting factor for the highly aerobic organism. The tubes were incubated with constant shaking at 24°C for 5 days. Final pH (Beckman pH meter, glass electrode) and visual estimation of turbidity were determined for each tube at that time.

The same procedure was used to determine the growth at different temperatures, except that the tubes slanted at 45° were shaken gently and uniformly by hand 4 times daily throughout the series. The triplicate tubes were incubated for 5 days in constant-temperature water baths.

Forty-nine modifications of Czapek's medium were prepared and each adjusted to pH 6.5. The sterilized media were dispensed aseptically in 3-ml portions into 4 medium-sized test tubes. Three tubes of each group were seeded with 0.1 ml of the standardized inoculum, inclined at 45° on the shaker, and incubated at 24°C with continuous shaking. At 2, 4, 6, and 12 days, the pH was determined, and turbidity was determined with a Fischer Nephelolofluorometer (430-mμ filter). The control tube of each group served as the blank for the turbidity reading.

RESULTS.—*Growth.*—The organism attained excellent growth in 5 days at pH levels varying from 4.5 to 8.0. Earliest maximal growth occurred at pH 6.5 in 4 days. There was some growth at pH 3.5 and pH 8.5 but none occurred below or above this.

Cells grew slowly at 5°C and quite well at 15, 20, 25, and 30°C. Growth was poor at 34° and absent at 37°. Turbidities eventually reached the same level at 5° and 10° as at the other temperatures, but such was not the case at 34°. Maximal turbidity was first attained at 25° in 4 days. This seems to support the opinion of Leukel and Martin (8) that the disappearance of smutted heads in the hot summer, even in the presence of ample tender new growth, results from growth inhibition of the parasite by the hot weather.

The smut fungus grew on many carbon sources (Table 1). Generally, the carbohydrates were good carbon and energy sources. Except for rhamnose, galactose, and lactose, the common monosaccharides and disaccharides tested supported extensive and

TABLE 1.—*Growth of S. cruenta in Czapek's liquid medium amended with a variety of carbon sources*[a]

Carbon source in lieu of sucrose[b]	Growth expressed as percentage absorption at indicated time		pH of medium at indicated time (initial pH = 6.5)	
	4 Days	6 Days	4 Days	6 Days
None	6	8	7.0	7.3
None and no $NaNO_3$	5	2	6.6	6.6
Glucose	60	91	6.6	7.0
Fructose	26	77	5.4	7.1
D-Mannose	59	90	6.7	7.5
D-Arabinose	13	44	4.9	6.5
Maltose	71	91	7.0	7.2
Sucrose	53	93	6.7	6.5
Trehalose	43	71	6.6	7.4
Raffinose	57	70	7.0	7.6
Melezitose	62	94	7.0	7.1
Dextrin	43	80	6.8	6.8
Glycogen	55	78	6.3	7.3
D-Ribose	12	40	5.2	6.8
Melibiose	13	15	6.3	6.8
Soluble Starch	37	35	6.9	6.9
Inulin	21	32	7.0	7.0
Inositol	10	17	6.4	6.9
Na Acetate	7	35	7.2	8.5
DL-α-Alanine	14	16	6.7	7.5
L-Glutamic Acid	8	20	6.9	7.5
Galactose	14	13	5.9	6.4
D-Xylose	8	6	5.0	5.6
D-Sorbitol	10	26	7.0	7.2
D-Mannitol	5	14	6.3	6.9
Na Lactate	5	12	6.5	6.8
Na Succinate	12	15	6.4	7.2
DL-Isoleucine	8	10	6.6	7.6
L-Aspartic Acid	5	8	7.3	7.9

[a] Carbon sources are grouped in descending order of visual turbidity at end of 12 days. Galactose through Aspartic acid supported only moderate growth even at 12 days: ++ as compared to ++++ for glucose.

[b] The following compounds resulted in very poor growth at end of 12 days: glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, dulcitol, pyruvate, glycine, valine, leucine, serine, methionine, tryptophane, and arginine.

The following compounds did not support growth: L-rhamnose, Na α-ketoglutarate, Na malate, Na citrate, threonine, cystine, cysteine, phenylalanine, tyrosine, dihydroxyphenylalanine, proline, histidine, and ornithine.

rapid growth. Trehalose, raffinose, and melezitose were likewise excellent. Two of the pentoses, arabinose and ribose, supported moderate growth. The organism was able to utilize the polysaccharides tested.

The sugar alcohols were not well utilized. Cells grew very slowly, and maximum growth attained was low. Various intermediates of the glycolytic and Kreb's cycle were not metabolized, with the exception of succinic acid, which supported slow growth. This behavior probably indicates only that the cells are impermeable to these acids under the conditions of the experiment (pH). Organic acids are absorbed by cells much better when they are undissociated (4).

The ability of the organism to utilize the amino acids for growth was variable, but at best they were little better than controls with no nitrogen source. Isoleucine, α-alanine, and glutamic acid supported a small amount of growth, but aspartic acid, arginine, and ornithine supported little more than did the controls in which there was no carbon source. Other amino acids supported no growth. After 12 days' incubation, the tubes containing α-alanine or glutamic acid showed relatively heavy growth comparable to that in tubes containing ribose or soluble starch. Isoleucine and aspartic acid produced growth comparable with xylose and sorbitol, whereas glycine, valine, leucine, serine, methionine, tryptophane, and arginine each supported some late growth. Such late growth probably resulted from selection of spontaneously occurring mutant organisms that were able to utilize the substrate, rather than an adaptation of all organisms to the substrate.

The various compounds fell into rather well-defined groups with respect to the ability to support growth of the organisms (Table 1). Some compounds supported rapid and continuous growth until a maximum was attained. This was the case with glucose, mannose, fructose, maltose, sucrose, raffinose, melezitose, trehalose, glycogen, dextrin, and soluble starch. Other compounds, such as galactose, inulin, and succinate, supported a slow but steady growth. Arabinose, ribose, the sugar alcohols, melibiose, acetate, α-alanine, and glutamic acid initially supported very slow growth, but after several days growth became rapid. Most of the amino acids, xylose, and lactate supported slow growth, but growth started slowly and late.

The changes in pH during growth in most of the carbohydrate-containing media were characteristic and were related generally to the number of cells in the culture at any given time. The pH dropped during the first 2 days and then increased to pH 6.4–7.0 as turbidity increased. Although the maximal turbidity varied in the different cultures, the pH was 6.8–7.6 at the time maximal growth was first attained. Thereafter, it dropped again, sometimes as low as 5.5. The smaller the amount of total growth produced, the less was this final drop in pH.

Generally, the utilization of organic acids resulted in an increasing pH with time. This was also the case with the amino acids, probably as a result of the production of ammonia or basic amines. It should be noted that in the absence of detectable growth there may be pH changes caused by the metabolism of the organisms.

No growth was produced in the absence of a carbon source. There was some growth in Czapek's medium from which a nitrogen source was omitted, but this probably resulted from contaminating nitrogen sources contained in the inoculum.

Oxidative metabolism.—Many experiments were involved, and it is very difficult to make direct comparisons of the results of the different experiments, since endogenous respiration varied from experiment to experiment. Endogenous QO_2 values for each experiment were deducted from those of the various substrates, and the adjusted QO_2 values make it pos-

TABLE 2.—*Oxygen uptake by mycelial cells and by yeast-like cells*

Substrate	Adjusted QO_2 values[a]	
	Mycelial cells	Yeast-like cells
D-Mannose	4.2	4.2
Trehalose	4.0	4.4
Melezitose	4.0	
Glucose	3.5	4.2
Sucrose	3.4	3.5
Fructose	3.2	3.3
Maltose	3.1	1.6
D-Arabinose	3.1	
Raffinose	3.0	3.1
Melibiose	2.8	
Dextrin	2.8	1.0
DL-Lysine . HCl	2.8	
L-Histidine . HCl	2.8[b]	2.5[b]
DL-Ornithine	2.6	
D-Xylose	2.6[b]	2.8[b]
DL-Serine	2.6	
L-Proline	2.6[b]	2.9[b]
DL-Isoleucine	2.5	
Na Acetate	2.4[b]	2.4[b]
Inositol	2.4	
DL-α-Alanine	2.2	1.8
Glycine	2.1	
L-Leucine	2.0	
L-Asparatic acid	2.0	
L-Arginine . HCl	2.0[b]	2.2[b]
Ca Gluconate		2.5[b]
Na Pyruvate	2.1	1.1
Phenylalanine	1.2	1.8
Ca Lactate	2.5	
DL-Threonine	1.8	
L-Glutamic acid	1.7	

[a] Endogenous rate deducted in each case. Endogenous QO_2 ranged from 1.4 to 2.8 (average 1.9) in different experiments; QO_2 for glucose in the same experiments were from 5.3 to 7.7 (average 6.1); QO_2 was 1.0–1.5 for Ca 2-ketogluconate, Na citrate, Na malate, glucose-6-phosphate, D-ribose, dulcitol, DL-valine, DL-methionine, dihydroxyphenylalanine, and thymine; QO_2 was 0.2–0.9 for galactose, lactose, inuline, mannitol, fructose-6-phosphate, Na α-ketoglutarate, Na succinate, and L-tyrosine; sorbitol, soluble starch, glycogen, cystine, L-cysteine, D-tryptophane, xanthine, adenine . SO_4 were not respired.

[b] Induced enzyme formation (See Table 3).

sible to compare the respiration upon the entire group of compounds.

In the experiments with mycelial cultures, no amino acid was oxidized nearly as rapidly as was glucose or sucrose (Table 2). Mannose was the most rapidly oxidized substrate; trehalose, melezitose, glucose, sucrose, fructose, maltose, arabinose, and raffinose were oxidized only slightly less rapidly. Lactose, starch, glycogen, sorbitol, adenine, xanthine, cystine, cysteine, and tryptophane were not utilized. Galactose, rhamnose, mannitol, inulin, fructose-6-phosphate, α-keloglutarate, succinate, and tyrosine were oxidized very slowly.

Similar results were obtained with the sporidial cultures (Table 2), with the exception of maltose and dextrin. These were oxidized at a much slower rate by the sporidial than by the mycelial cultures. Generally, the sporidial cultures were slightly more active than the mycelial cultures in oxidizing the various utilizable substrates, but this may have been a result of their having been grown on a richer medium than were the latter cultures.

The results are combined and arranged in order of decreasing rate of oxygen uptake for the mycelial cultures with equivalent values for the adjacent sporidial cultures (Table 2).

A number of compounds, *viz.* xylose, pyruvate, acetate, Ca gluconate, Ca 2-ketogluconate, arginine, histidine, phenylalanine, and proline, exhibited utilization-rate curves such as are observed in induced enzyme formation (3, 9, 10). In these cases (Table 3) there was a lag period, variable with each compound, during which the rate of oxygen uptake did not exceed that of the endogenous rate. This was followed by a gradually increasing rate until it became constant. This constant rate was achieved within 1 hour with proline, but only began to be attained in 3 hours with phenylalanine (Table 3).

DISCUSSION.—The limited amount of growth on Czapek's medium from which a nitrogen source was omitted probably resulted from contaminating nitrogen materials contained in the inoculum rather than from nitrogen fixation. Cells grown in nitrogen-containing medium can store, in all probability, sufficient nitrogen to enable the cells to effect some growth when they are transferred to a nitrogen-deficient medium. Teliospores can germinate, produce some mycelium, and fuse with other adjacent mycelium, or promycelium, in distilled water, and the organism can grow through its entire life cycle from teliospore to teliospore in a simple inorganic medium with sucrose as the energy source (2).

Growth is distinctly stimulated by a rich medium, such as Difco nutrient broth, or by the addition of yeast extract to Czapek's medium. This may be due to some growth factor (organic or inorganic) or simply to an optimal combination of nitrogen and carbon sources. It is evident, however, that the organism is able to synthesize all needed nitrogen compounds from sodium nitrate and all of its carbon requirements from any one of a wide variety of organic compounds. A very complex series of enzyme systems are required to effect these reactions. The organism is relatively independent of many of its environmental surround-

TABLE 3.—*Oxygen uptake by yeast-like cultures[a] of S. cruenta at hourly intervals*

Substrate	Adjusted QO_2 at indicated hour[b]			
	1	2	3	4
D-Xylose	0.9	1.6	2.8	2.8
Na Pyruvate	0.0	0.4	0.9	1.1
Na Acetate	0.0	0.8	1.8	2.4
Ca Gluconate	0.0	0.1	1.2	2.5
Ca 2-Ketogluconate	0.0	0.3	0.6	1.1
L-Proline	0.3	0.8	2.6	2.9
L-Histidine . HCl	0.0	0.4	1.6	2.5
L-Arginine . HCl	0.0	0.2	1.4	2.2
DL-Phenylalanine	0.0	0.0	0.6	1.8

[a] Behavior was similar with mycelial suspensions.

[b] QO_2 with endogenous deducted in each case.

ings.

With such simple and uncomplicated growth requirements, the host specificity of this fungus would appear to have some basis other than nutritive. Certainly if the spores can germinate and produce mycelia that fuse, all in distilled water, nutrition cannot very well be the limiting step in the infection of a species of plant other than the sorghums.

Except for polysaccharides and a few other exceptions, the compounds that supported growth also were readily oxidized, and the converse also was true. But for dextrin, which was utilized by mycelial cultures but not by the yeast-like form, the polysaccharides were not respired although they supported growth quite well. Under conditions where growth can occur, and where the inoculum is not washed (thereby removing extracellular hydrolytic enzymes), the cells perhaps can hydrolyze and, therefore, utilize the polysaccharides after they are degraded.

Induced or adaptive enzyme formation probably occurred on a number of the substrates, e.g. pyruvate, acetate, gluconate, 2-ketogluconate, proline, histidine, arginine, and phenylalanine. These results, however, must be taken only as *prima facie* evidence of induced enzyme formation for these compounds. A great many factors may alter this type of behavior (4, 12).

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PHYTOPATHOLOGICAL NOTES

Occurrence of the Bacterial Wilt Organism in Alfalfa Seed.[1] M. W. Cormack and J. E. Moffatt. Bacterial wilt of alfalfa, caused by *Corynebacterium insidiosum* (McCull.) H. L. Jens., has become prevalent in many of the alfalfa-growing regions of North America during the past 25 years. This important disease has spread erratically, as shown by its frequent appearance in areas far from any known source of infection. Seed transmission is a possible explanation for the introduction of the organism into new areas, but it has not been previously proved. Jones[2] made a thorough study of the development of the bacteria and, by staining and isolation, traced them in the upper parts of infected seed-bearing alfalfa plants. He demonstrated their presence in the base of a seed pod of 1 plant, but did not find them in the seed. Peltier and Jensen[3] failed in many attempts to isolate the organism from seed obtained from diseased plants.

In this investigation, histological, isolation, and infectivity studies were made on many collections of seed and other parts of alfalfa plants infected with bacterial wilt. Since severely diseased plants rarely produced seed, most of the field samples were obtained from plants with slight to moderate infection. Special material for isolation and histological examination was obtained in the greenhouse by hand tripping and cross pollination of flowers of moderately infected plants.

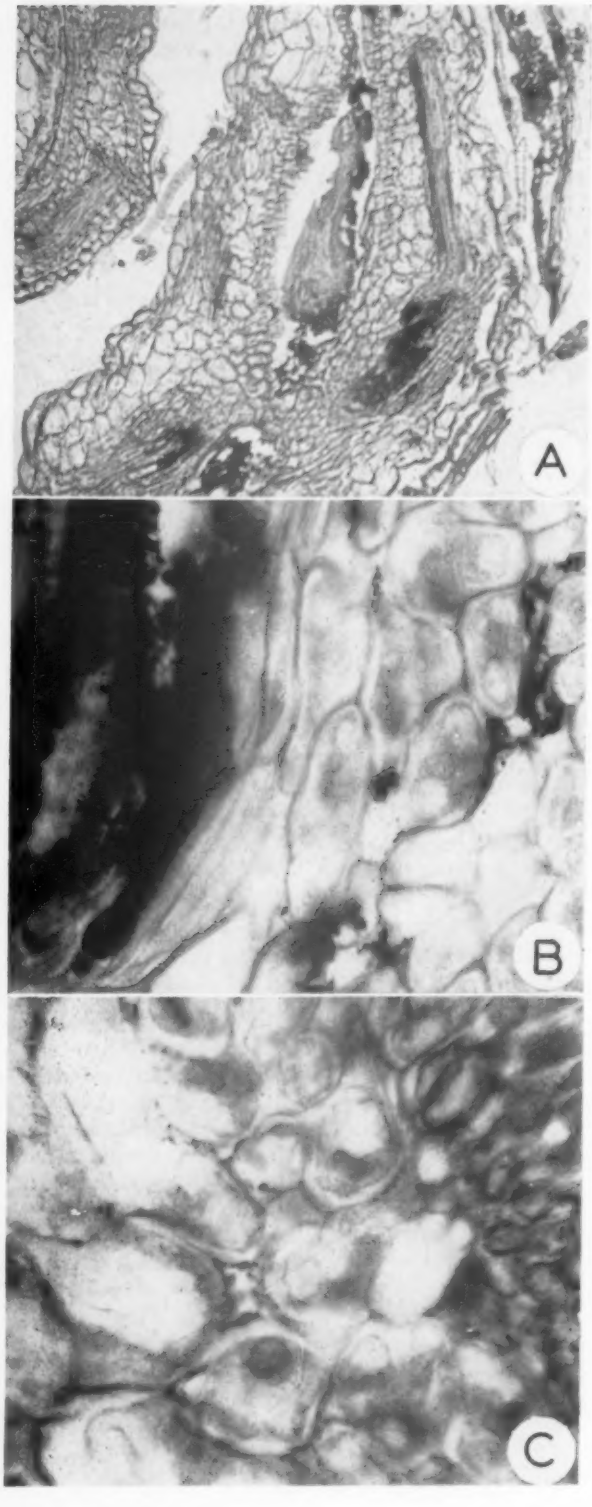
A histological examination was made of the seed-bearing portions of infected plants at various stages of development. Sections 12μ thick were stained by

[1] Contribution No. 1515 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

[2] Jones, F. R. 1928. Development of the bacteria causing wilt in the alfalfa plant as influenced by growth and winter injury. Jour. Agr. Res. 37: 545-569.

[3] Peltier, G. L., and J. H. Jensen. 1930. Alfalfa wilt in Nebraska. Nebr. Agr. Exp. Sta. Bul. 240, 35 p.

a combination of Conant's quadruple stain and Nyfeld's modification of Gram's method for differential staining of bacteria. The gram-positive cells of *C. insidiosum* were observed in varying numbers in the vascular system of the flowering rachis and pedicel portions of all plants studied. They were not observed in the flowers before fertilization but were found in the vascular and parenchymatous tissues of several seed pods (Fig. 1, A, B). Normal seeds seldom developed when bacteria were numerous in the pedicel or in the basal portion of the seed pod. Masses of bacteria were noted also in the vicinity of apparently aborted seeds (Fig. 1, A). The bacteria were relatively scarce within the seed, even when they were fairly abundant in the pedicel and pod. In the immature seed, they occurred mainly in the intercellular spaces of parenchymatous tissues below the Malpighian cells (Fig. 1, C). A few observations made on mature infected seed indicated that the bacteria were confined mainly to the aleurone layer of the endosperm. They were not observed in the cotyledons.



A few typical cultures of *C. insidiosum* were isolated from alfalfa seed produced by diseased plants. Most good seeds did not yield the organism; it was obtained more readily from discolored and shrivelled seeds.

The bacterial wilt organism was most easily detected in seeds and other plant materials by inoculations to healthy alfalfa plants. The seeds were thoroughly cleaned, surface disinfected in sodium hypochlorite (2 per cent chlorine), aseptically pulverized, and soaked in sterile water for 4 hours. A small portion of this material was aseptically inserted into a wound about 0.5 in. below the crown of each of 30 disease-free plants. These plants were grown in sterilized soil in the greenhouse for 12 weeks after inoculation and then examined for the root and top symptoms of bacterial wilt. In a representative test of inocula prepared from parts of a moderately diseased plant, those from the upper rachis and pedicel portions caused infection of 87 per cent of the healthy plants inoculated; those from the pod, 77 per cent; those from discolored and shrivelled seed, 70 per cent; and those from sound plump seed, 23 per cent. No infection occurred in wounded noninoculated control plants.

The bacterial wilt organism apparently does not occur commonly in seeds of alfalfa. It was detected with difficulty in selected material in the histological and isolation studies, and it was not found in many random samples of seed grown in areas infested by the wilt organism. Diseased plants generally produce relatively few seeds, however, and they would be very difficult to detect in large lots of seed. The low-

FIG. 1. Photomicrographs of longitudinal sections of seed pod and seed of alfalfa infected with *C. insidiosum*. A) Masses of bacteria in the base of a pod and in the vicinity of a poorly developed seed (near center). ×90. B) Bacteria in vessels and between parenchyma cells in the base of a pod. ×575. C) Bacteria between the cells and in the intercellular spaces below the Malpighian layer (upper right) in an immature seed. ×770.

quality seed often produced by diseased plants and the plant debris in poorly cleaned samples apparently carry the organism to a much greater extent than does good seed. These results indicate that alfalfa seed grown in areas infested by the bacterial wilt organism can be an important factor in spreading the disease into new areas or new fields. A few infected seeds or a small quantity of infested debris in a seed sample probably would be sufficient to introduce the organism into the soil and to initiate infection.—Plant Pathology Section, Science Service Laboratory, Lethbridge, Alberta, Canada.

Factors Affecting Apothecial Production by Sclerotinia sclerotiorum. LAURENCE H. PURDY.[1] While making a taxonomic study of a number of isolates of *S. sclerotiorum* (Lib.) d By. emend Purdy[2], the author encountered a marked difference in the ability of different isolates to form apothecia under environmental conditions reported to be near optimum[3]. Some isolates formed apothecia readily and rapidly, whereas others formed them slowly and sporadically or not at all. For example, 3 isolates of *S. sclerotiorum* "major" produced initials in 45, 25, and 16 days, respectively, from sclerotia recently produced in potato-dextrose agar (PDA) plates, whereas other isolates of *S. sclerotiorum* "minor" did not produce initials under near-optimum conditions even after 90 days.

Most of the previous studies concerning the environmental conditions that influence apothecial production have been concerned with the influence of environment on development and expansion of the apothecial discs. Henson and Valleau[3] reported that constant moisture, a temperature of 14°C, and sufficient time of exposure were important factors in the initiation of stipes of *S. sclerotiorum* and *S. trifoliorum*. Björling[4] has shown that light is necessary for the expansion of the discs of *S. trifoliorum* and that light influences the length of the apothecial stipes. Coe[5] reported that artificial light hindered or inhibited the normal development of apothecia of *S. trifoliorum*.

TABLE 1.—*Length of time elapsing before apothecial initials were formed by sclerotia of Sclerotinia isolates produced and aged at different temperatures for different lengths of time*

Sclerotial type	Days aged	Days before apothecial initials appeared[a] from sclerotia produced at indicated temperature (°C)						
		4°	12°	15°	18°	21°	24°	27°
Large	30	36	45	48	37	40	43	—
	60	23	8	7	7	8	20	27
	90	27	9	7	9	10	45	—
Small	30	35	40	43	47	—	—	—
	60	23	27	39	78	74	70	73
	90	31	28	37	90	—	—	—

[a] Following the ageing period, sclerotia were placed in distilled water at 15°C. A broken dash indicates that no initials appeared after 150 days.

The present study was concerned with the effect of aging of sclerotia and the effect of temperature and substrate upon which sclerotia were produced on their subsequent ability to produce apothecia.

Two isolates, representatives of a large- and a small-sclerotium type, were selected and allowed to produce sclerotia on PDA at 4°, 12°, 15°, 18°, 21°, 24°, and 27°C. After sclerotia were produced at the different temperatures, they were allowed to remain on the agar surface at their respective temperatures for 30, 60, or 90 days before they were placed in near-optimum conditions for apothecial production. The large-sclerotium type produced and aged at 15° or 18° for 60 or 90 days produced apothecial initials within 7 days after they were placed in water at 15° (Table 1). In general, initials formed more rapidly from sclerotia of the large-sclerotium type produced at 12–24° than from those produced at 4° or 27°. The shortest time in which initials appeared from the small-sclerotium type was 23 days from sclerotia that had been produced and aged at 4° for 60 days.

The advantages of natural media over artificial media for the culture and fructification of certain fungi have been pointed out by Snyder and Hansen[6]. Sclerotia of a small-sclerotium type that had been produced on autoclaved celery or PDA at 4°, 12°, 18°, and 24°C were compared as to their ability to form apothecia when placed in distilled water at 15°. Very few initials were produced by sclerotia produced on PDA at any temperatures tested or from sclerotia produced on autoclaved celery at 18° or 24° (Fig. 1). Apothecial initials were produced 10–15 days sooner from sclerotia produced on autoclaved celery at 4° and 12° than from sclerotia produced at 18° or 24°.

In agreement with Björling[4], light was found necessary for the normal development and expansion of apothecial discs but not for the formation of initials. After initials had appeared, mature expanded apothe-

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Former Research Assistant, University of California, Davis, California, now Pathologist, U. S. Department of Agriculture, Agricultural Research Service, Field Crops Research Branch, Pullman, Washington.

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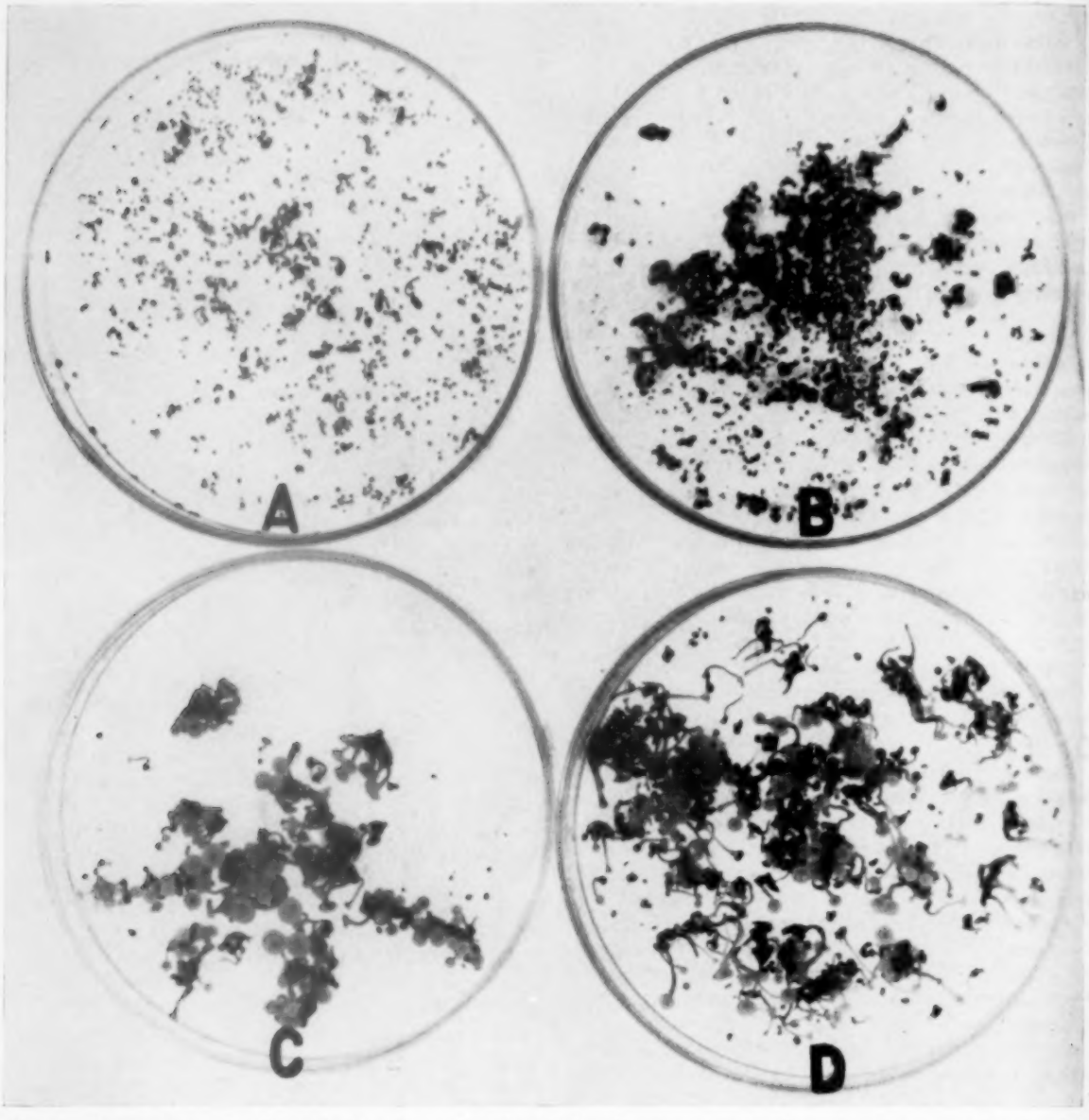

FIG. 1. Increased apothecial production from sclerotia of *S. sclerotiorum* "minor" produced on autoclaved celery in comparison with that from sclerotia produced on PDA. A, B) Sclerotia produced on PDA; A) at 4°, B) at 12°. C, D) Sclerotia produced on autoclaved celery; C) at 4°, D) at 12°. Apothecial initials induced at 15°C.

cia were formed by all isolates studied when exposed to either artificial or natural light. This finding is in disagreement with Coe's report[5] that artificial light inhibited the development of apothecia of *S. trifoliorum* (a synonym of *S. sclerotiorum*[2]).

Abnormal apothecia were occasionally produced by most of the isolates; however, single ascospores from these abnormal apothecia grew and produced normal sclerotia that in turn produced normal apothecia, indicating that the abnormalities were not due to genetic mutations.—University of California, Davis, California.